

Attorney Docket No.: CST-138 CIP2

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
APPLICATION FOR UNITED STATES LETTERS PATENT

INVENTOR(S): Michael J. Comb
Yi Tan
Hui Zhang

TITLE: Production of Motif-Specific and Context-Independent Antibodies Using Peptide Libraries as Antigens

ATTORNEY: JAMES GREGORY CULLEM
Intellectual Property Counsel
CELL SIGNALING TECHNOLOGY, INC.
166B Cummings Center
Beverly, MA 01915
Telephone: (978) 867-2311

Express Mail Label No.: EE742586909US

Date of Deposit: November 13, 2001

PRODUCTION OF MOTIF-SPECIFIC AND CONTEXT-INDEPENDENT ANTIBODIES USING PEPTIDE LIBRARIES AS ANTIGENS

RELATED APPLICATIONS

This application is a continuation-in-part of, and claims priority to, 5 USSN 09/535,364, filed March 24, 2000, which is a continuation-in-part of USSN 09/148,712, filed September 4, 1998, both presently pending, the teachings of which are incorporated by reference herein.

BACKGROUND OF THE INVENTION

10 The present invention relates to the production of motif-specific, context-independent antibodies which are specific to a short amino acid motif presented in the context of variable surrounding amino acid or peptide sequences. Antibodies with these properties are useful in characterizing various forms of cellular regulation as well as serving to profile genome wide changes in cellular protein levels and protein 15 modification.

Identifying the targets of intracellular signaling cascades are of major importance in understanding cell growth, differentiation, and cell death. Protein kinase cascades relay information from the cell surface to multiple cellular compartments including the nucleus and more distant cell 20 processes such as synapses (Karin *et al.*, Curr. Opin. Cell. Biol. 6:415-424 (1994)). Although a few targets of protein phosphorylation have been identified, most remain unknown, particularly those that regulate cell growth and differentiation. For example, the MAP kinase cascade is known to play an important role in the regulation of cell growth (Lewis *et al.*, Adv. Cancer Res. 74:49-139 (1998), Crowley *et al.*, Cell 77:841-852 25 (1994)). However, beyond a handful of substrates, few protein targets responsible for the diverse actions of the MAP kinase cascade have been

identified (Fukunaga and Hunter, *EMBO* 16(8):1921-1933 (1997),
Stukenberg *et al.*, *Curr. Biol.* 7:338-348 (1997)).

Another example of cell signaling proteins are the 14-3-3 proteins,
which represent a phylogenetically conserved family of phosphoserine
5 binding proteins whose precise role in cell signaling has yet to be
determined (Burbelo and Hall, *Curr. Biol.* 5(2):95-96 (1995)). These
proteins represent a large fraction of total brain protein and are known to
bind a wide variety of signaling molecules including: ras, raf, bad, cdc25,
and many others (Yaffe *et al.*, *Cell* 91:961-971 (1997)). Recently, it has
10 been shown that 14-3-3 proteins bind specifically to phosphorylated sites
on proteins with the following motif: RXRSXS*XP where S* is
phosphoserine and X represents any amino acid (Muslin *et al.*, *Cell*
84:889-897 (1996), Yaffe *et al. supra*(1997)).

Similarly, histones have long been known to be modified by
15 acetylation at specific lysine residues. Acetylation of lysine in histones is
thought to reduce protein-DNA interactions and serve to open chromatin
in regions undergoing transcription (Struhl, *Genes & Development*,
12:599-606 (1998)). Recently, other proteins associated with transcription
complexes have been shown to be acetylated on lysine although the
20 functional significance is unclear (Imhof *et al.*, *Curr. Biol.* 7:689-692
(1997), Struhl *supra* (1998)).

Antibodies against phosphotyrosine have proven to be of great
value in identifying and characterizing intracellular signaling mechanisms
(Ross *et al.*, *Nature* 294:654 (1981), Kozma *et al.*, *Method. Enzymol.*
25 201:28 (1991), White and Backer, *Method. Enzymol.* 201:65 (1991),
Kamps, *Method. Enzymol.* 201:101 (1991)). Their value derives from two
properties; 1) their ability to discriminate whether or not a protein is
tyrosine phosphorylated, and 2) their ability to react with a large variety of
different proteins. These properties have proven invaluable in tracing

intracellular signaling pathways and identifying new targets of activated tyrosine kinases.

Ideally, the most useful phosphotyrosine antibodies should be as general as possible, that is they should recognize phosphotyrosine independently of the protein sequences in which it is embedded (context independent) so as to allow detection of all possible phosphotyrosine residues. The most successful approaches for producing phosphotyrosine antibodies have utilized phosphotyrosine or phosphotyramine coupled via their free amino groups to keyhole limpet hemocyanin using hetero- or bifunctional crosslinking agents (Frackelton *et al.*, *Method. Enzymol.* 201:79 (1991), White and Backer *supra* (1991), Wang, *Method. Enzymol.* 201:53 (1991), Kamps *supra* (1991)). Although currently produced polyclonal and monoclonal phosphotyrosine antibodies do recognize many different proteins, they often show crossreactivity with other phosphate containing compounds, for example, mononucleotides (Frackelton *et al. supra* (1991), Kamps *supra* (1991)). More importantly, most phosphotyrosine antibodies raised in this fashion display variable sequence reactivity, depending not only on the phosphorylated amino acid, but also upon the amino acid sequences surrounding phosphotyrosine. For example, the present inventors have observed that most phosphotyrosine antibodies do not recognize phosphotyrosine preceded by proline as found in the activation loop of JNK and hence do not react significantly with activated (tyrosine phosphorylated) JNK [(Tan *et al.* unpublished observations)]. The reason for the variable reactivity is likely due to the fact that the phosphotyrosine antigen is not presented directly to the immune system in the context of variable surrounding amino acids, but is instead presented as a hapten, inappropriately coupled to the KLH carrier via artificial linkages. This approach tends to produce antibodies that react well with phosphotyrosine but are sometimes blocked by surrounding amino acids as they are not present in the antigen.

Other approaches have utilized total cellular phosphotyrosine containing proteins as immunogens (Glenney, *Method. Enzymol.* 201:92 (1991), Wang *supra* (1991)) with considerable success but the context-dependence of the resulting antibody specificities was not carefully
5 determined, although antibodies raised in this fashion did react with a majority of tyrosine phosphorylated proteins. Estimates as to the fraction of tyrosine phosphorylated proteins detected range from 50% to 94% (Kamps *supra* (1991)).

Attempts to use the above mentioned techniques to produce
10 similar antibodies for phosphoserine and phosphothreonine have met with limited success. Antibodies produced to date have limited crossreactivity and lower affinity for phosphoserine or phosphothreonine probably due to the poor immunogenicity of these phospho-amino acids compared to phosphotyrosine (Heffetz *et al.*, *Method. Enzymol.* 201:44
15 (1991)). Context-dependence and low affinity have limited the utility of currently available phosphoserine and phosphothreonine antibodies, especially when compared to phosphotyrosine antibodies.

Site-specific phosphoserine and phosphothreonine antibodies were first described by Nairn *et al.* in 1982 and have proven to be highly
20 useful tools to study protein phosphorylation (Czemik *et al.*, *Method. Enzymol.* 201:264 (1991), Czernik *et al.*, *Neuroprot.* 6:56-61 (1995)). One drawback of this type of antibody is that a different antibody needs to be produced for each site of interest. Clearly, development of antibodies that detect phosphoserine or phosphothreonine in a substantially context-
25 independent fashion would be desirable for use in tracing serine/threonine kinase cascades and in defining their biological responses. Likewise, development of context-independent phosphotyrosine antibodies would overcome the limitations of currently available antibodies.

Motif-specific, context-independent antibodies would also be useful in identifying new targets of 14-3-3 action (*i.e.*, other proteins phosphorylated at this motif) and in characterizing the protein kinases that phosphorylate these sites. Likewise antibodies reactive against acetylated lysine would serve as useful tools to study the functional significance of acetylation of histones.

Such antibodies can further be used as general reagents for detecting phosphorylation or other enzymatic modification *in vitro*, such as in high throughput kinase assays for drug screens, as a single antibody can be used to recognize many different phosphorylated substrates. Phosphotyrosine antibodies are currently employed in high throughput kinase assays to screen for selective, high affinity tyrosine kinase inhibitors. Compounds or drugs that block enzyme activity are detected by their ability to inhibit kinase activity as determined by a reduction of phosphotyrosine antibody binding to phosphorylated substrate. Similar assays can be set up to screen for pharmaceutically useful compounds using antibodies produced as described above for phosphoserine, phosphothreonine, or antibodies detecting other protein modifications.

Antibodies that detect short recurring motifs in a context-independent fashion will also be particularly useful in profiling genome wide changes in protein levels and protein modification. For example, the use of context-independent phosphothreonine antibodies and 2D gel electrophoresis to profile genome wide changes in protein phosphorylation (Patterson and Garrels, Cell Biology: A Laboratory Handbook 249-257 (1994), Academic Press) as the result of drug treatment or overexpression of a particular protein will undoubtedly prove useful in identifying potential drug-protein interactions and suggest new downstream targets for overexpressed proteins.

SUMMARY OF THE INVENTION

In accordance with the present invention, there is provided a method of producing antibodies that selectively recognize a plurality of peptides or proteins within a genome that contain the same short motif.

5 Motif-specific antibodies of the invention therefore recognize the motif highly independent of the surrounding amino acid, peptide, or protein sequences. The method allows the production of motif-specific, context-independent antibodies that recognize single modified amino acids, for example phosphorylated serine, threonine, and tyrosine, or acetylated

10 lysine, as well other unmodified or modified short motifs of multiple invariant amino acids.

The method encompasses the production and purification of highly context-independent antibodies that recognize specific and highly degenerate amino acid motifs common to multiple peptides or proteins

15 within a genome, such as those found in kinase consensus sequences or other enzyme binding sites. Motifs recognized by the antibodies of the invention typically comprise one to six invariant amino acids. Furthermore, the method can be used to produce highly context-independent polyclonal or monoclonal antibodies.

20 Antibodies produced by the method of the present invention may be specific to virtually any desired recurring protein motif, either modified or unmodified. In preferred embodiments, the motif includes at least one modified amino acid. For example, the method can be used to produce antibodies recognizing phosphothreonine alone or phosphothreonine

25 together with several invariant amino acids in a motif, such as found in MAPK substrates, 14-3-3 binding proteins, or CDK consensus phosphorylation sites. It can also be used to produce antibodies specific for other modified amino acids, for example, acetylated lysine or nitrotyrosine, or to detect any short non-unique motif of one or more

30 amino acids, in a highly context-independent fashion. Alternatively, the

antibodies may be specific for unmodified motifs, such as those found in protein cleavage motifs, e.g. caspase cleavage motifs.

The invention also provides motif-specific, context-independent antibodies that specifically recognize short motifs comprising all or part of the following: MAPK consensus substrate motifs, CDK consensus substrate motifs, PKA consensus substrate motifs, Akt consensus substrate motifs, PKC consensus substrate motifs, ATM consensus substrate motifs, 14-3-3 consensus binding motifs, PDK1 consensus docking motifs, phosphothreonine-X-(arginine(R)/lysine(K)), PKC Zeta consensus substrate motifs, ABL kinase consensus substrate motifs, insulin receptor consensus substrate motifs, PI3K P85 consensus binding motifs, CaMKII consensus substrate motifs, SRC kinase consensus substrate motifs, CDC2/CDK2 consensus substrate motifs, GSK3 kinase consensus substrate motifs, and proline(P)-(phosphoserine/phosphothreonine)-proline(P). The antibodies of the invention are not limited, however, to these exemplary motifs.

The present invention further provides a method of profiling large and diverse protein populations on a genome-wide scale by utilizing motif-specific, context-independent antibodies against motifs conserved on such proteins. For example, phosphorylation-specific antibodies allow genome-wide profiling of changes in protein phosphorylation as a result of drug treatment.

The present invention also provides a method of identifying an unknown substrate of a known enzyme through the use of motif-specific, context-independent antibodies which are raised against motifs common to other substrates of the enzyme.

The use of such motif-specific, context-independent antibodies as a reagent for the detection of enzymatic modifications of a given motif within a substrate is also encompassed by the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1a is a table depicting the specificity of the affinity-purified, polyclonal antibodies produced against a phosphorylated threonine peptide library in Example I, when tested against specific peptides (SEQ ID NOs: 1-13).

Figure 1b is a table depicting the specificity of the phosphothreonine antibodies of Example I when tested against various phosphopeptide libraries (SEQ ID NOs: 14-29).

Figure 1c is a Western analysis which depicts the reactivity of the phosphothreonine antibodies of Example I against cell extracts from cells treated with and without okadaic acid and against other phosphoproteins.

Figure 1d is a table depicting the context-independence of the anti-phosphothreonine antibodies of Example I as shown by immobilized grid.

Figure 2a is a table depicting the specificity of the affinity-purified, polyclonal antibodies produced against a phosphorylated PXS*P peptide library in Example II (SEQ ID NOs: 30-32).

Figure 2b is a Western analysis depicting the reactivity of the phospho-PXS*P antibodies of Example II against cell extracts from cells treated with and without okadaic acid and against other phosphoproteins.

Figure 3a is a table depicting the lack of reactivity of the affinity-purified, polyclonal 14-3-3 antibodies of Example III when tested against non-phosphopeptides or phosphopeptides lacking the motif (SEQ ID NOs: 33-40).

Figure 3b is a Western analysis depicting the reactivity of the phospho-14-3-3 antibodies of Example III against cell extracts from cells transfected with GST-Bad and with TPA.

Figure 4a is a table depicting the specificity of the monoclonal antibodies produced against a phosphorylated PXT*PXR library in Example IV (SEQ ID NOs: 41-44).

5 Figure 4b is a Western analysis depicting the reactivity of the CDK consensus site monoclonal antibodies of Example IV against phosphorylated and nonphosphorylated RB protein.

Figure 5a is a Western analysis depicting the specificity of the acetylated-lysine antibodies of Example V against acetylated BSA.

10 Figure 5b is a Western analysis depicting the reactivity of the acetylated-lysine antibodies of Example V against various proteins present in C6-cell extracts when antibodies are preincubated with nonacetylated peptide library.

15 Figure 5c is a Western analysis depicting the reactivity of the acetylated-lysine antibodies of Example V against various proteins present in C6-cell extracts when antibodies are preincubated with acetylated peptide library.

Figure 5d is a Western analysis depicting the reactivity of the acetylated-lysine antibodies of Example V against the control acetylated BSA when antibodies are preincubated with acetylated peptide library.

20 Figure 6 shows the signal to noise ratio of ELISA readings using phospho-Akt substrate antibody with phospho-peptides of Akt substrates vs. non-phospho-peptides of Akt substrates (SEQ ID NOs: 48-61).

Figure 7 is a Western analysis of calyculin A-treated A431 cells using phospho-Akt substrate antibody.

25 Figure 8 shows the signal to noise ratio of ELISA reading using phospho-PKA substrates antibody against peptides have arginine or lysine at -3 position (SEQ ID NOs: 62-77).

Figure 9 is a Western analysis of calyculin A-treated A431 cells using phospho-PKA substrates antibody.

Figure 10 is a Western analysis of A431 cell extracts phosphorylated by protein kinase A, ERK2 and CDC2/cyclinA *in vitro* using phospho-PKA substrate antibody.

Figure 11 shows the signal to noise ratio of ELISA reading using phospho-serine/threonine phenylalanine antibody against the peptides containing phenylalanine, tyrosine or tryptophan (SEQ ID NOs: 78-87).

Figure 12 is a Western analysis of calyculin A-treated A431 cells using phospho-serine/phenylalanine substrates antibody.

Figure 13 shows signal to noise ratio of ELISA reading, using a motif-specific, context-independent antibody specific for the phospho-PKC consensus substrate motif, with phospho-PKC substrate containing peptides and nonphospho peptides (SEQ ID NOs: 113-116).

Figure 14 is a Western blot analysis of WEHI 231 cells or Jurkat cells untreated (–) or treated (+) with TPA, probed with a motif-specific, context-independent antibody specific for the phospho-PKC consensus substrate motif.

Figure 15 is a Western blot analysis of whole cell lysates of Jurkat cells untreated and treated with PMA (50 ng/ml) and ionomycin (1 μ M) for 20 minutes prior to lysis, using a motif-specific, context-independent antibody specific for the phospho-PKC consensus substrate motif. Proteins were separated by 2D electrophoresis prior to blotting.

Figure 16 is a Western analysis of whole cell lysates of different cells untreated or treated with peroxynitrite, degraded peroxynitrite or pervanadate using a context-independent antibodies specific for

phosphotyrosine (upper), and a polyclonal context-independent antibody specific for nitrotyrosine (lower).

Figure 17 shows immunocytochemical staining of NIH/3T3 cells treated with degraded peroxynitrite (control) or peroxynitrite using a polyclonal context-independent antibody specific for nitrotyrosine (brown).

Figure 18 depicts phosphothreonine-X-arginine motif-specific antibody ELISAs: Signal to noise ratio of phospho versus nonphospho peptides containing the phospho-threonine-X-arginine motif. (T* denotes phosphorylated threonine) (SEQ ID NOs: 117-122).

Figure 19 is a Western blot analysis of Jurkat cell extracts untreated (–) and treated (+) with TPA or Calyculin A, using a motif-specific, context-independent antibody specific for the phosphothreonine-X-arginine motif.

Figure 20 shows immunohistochemical staining of proteins containing phosphorylated threonine-X-arginine motifs in paraffin-embedded human breast carcinoma, using a context-independent antibody specific for the motif.

Figure 21 is a Western blot analysis of calyculin A treated A431 cells, using a context-independent antibody specific for the phospho-14-3-3 binding motif #2 (phospho(Ser)-Arg-X-(Tyr/Phe)-X-pSer).

Figure 22 depicts phospho-ATM/ATR consensus substrate motif-specific, context-independent antibody ELISAs: Signal to noise ratio of phospho versus nonphospho peptides. (S* or T* denote phosphorylated serine or threonine) (SEQ ID NOs: 123-131).

Figure 23 shows Chk2 transfected and UV treated COS cell extracts immunoprecipitated with Chk2 antibody then detected by

Western blotting, using a motif-specific, context-independent antibody specific for phospho-ATM/ATR consensus substrate motif.

Figure 24 is a Western blot analysis of UV treated COS cells, using a context-independent antibody specific for phospho-ATM/ATR consensus substrate motif.

Figure 25 depicts phospho-14-3-3 binding motif-specific, context-independent monoclonal antibody ELISAs: Signal to noise ratio of phospho versus nonphospho 14-3-3 binding motif-containing peptides. (T* and S* denote phosphorylated threonine and serine) (SEQ ID NOs: 132-137).

Figure 26 is a Western blot analysis of calyculin A treated A431 cells, using antibodies specific for phospho-14-3-3 binding motif #1 (left, monoclonal; right, polyclonal).

Figure 27 depicts phospho-PDK1 docking motif-specific, context-independent monoclonal antibody ELISAs: Signal to noise ratio of phospho versus non-phospho peptides corresponding to potential PDK1 docking motifs. (T* and S* denote phosphorylated threonine and serine) (SEQ ID NOs: 138-145).

Figure 28 is a Western blot analysis of extracts from A431 cells untreated or treated with 0.1 μ M calyculin A for 30 minutes prior to lysis, using a monoclonal context-independent antibody specific for the phospho- PDK1 docking motif.

Figure 29 shows immunoprecipitation of extracts from NIH/3T3 cells untreated or treated with 100 ng/ml of PDGF for 20 minutes prior to lysis, using a monoclonal context-independent antibody specific for phospho-PDK1 docking motif and an Akt antibody, then probed with the

PDK1 docking motif monoclonal antibody (upper) and the Akt antibody (lower).

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based upon the concept that the concentration of any individual sequence in a peptide library used as antigen is extremely low and hence will be insufficient to drive an immune response in a host. The only antigenic determinants of sufficiently high concentration to drive the immune response are thus the fixed (*i.e.* invariant) residues common to each sequence, as well as the peptide backbone itself.

Immunizing a host with a degenerate peptide library comprising (i) a short fixed amino acid motif (the target motif) containing one or more invariant amino acids, and (ii) a plurality of degenerate amino acids representing many of the 19 amino acids (excepting cysteine) at positions flanking the motif will produce antibodies specific for all or part of the target motif and tolerant to many, if not all, amino acids at the degenerate (*i.e.* variable) positions flanking the fixed motif. Such antibodies will then react with the antigenic determinant (a motif consisting of all or part of the target motif) despite being presented in the context of a broad range of different surrounding amino acid, peptide, or protein sequences. The highly context-independent antibodies are thus capable of recognizing a plurality of peptides or proteins within a genome that contain the motif, typically consisting of 1-6 invariant amino acids. The produced antibody may be specific for a motif consisting of a part of the target motif that contains a modified residue, in which case the antibody will recognize peptides and proteins containing that part of the target motif, as well as proteins containing the entire target motif. The invariant residue(s) of the fixed target motif may be a single unmodified or modified amino acid, such as a phosphorylated or unphosphorylated residue, or may be

multiple unmodified or modified amino acids, such as a consensus recognition site, comprising a short motif. These short motifs, unlike longer peptide sequences that represent unique protein sequence or sites, frequently serve as targets of enzymatic modification, such as
5 single phosphorylatable residues or consensus substrate or consensus binding sites, which are common to multiple peptide or protein targets in a cell.

As used herein, "antibodies" means polyclonal or monoclonal antibodies, including Fc fragments, Fab fragments, chimeric antibodies,
10 or other antigen-specific antibody fragments.

As used herein, "degenerate amino acids" means amino acid positions that are non-fixed and thus variant. The term includes amino acid positions that are highly degenerate, representing most, if not all, of the 20 amino acids at that position, positions that are moderately
15 degenerate (*i.e.* biased towards certain of the 20 amino acids), and positions that are slightly degenerate, representing at least two different amino acids.

As used herein, "degenerate peptide library" means a peptide library comprising a plurality of individual peptides collectively containing
20 one or more degenerate amino acids. The term includes a peptide library of any length suitable for use as an immunogen to raise anti-peptide antibodies, typically, but not limited to, about 6 to 20 amino acids.

As used herein, "flanking," with respect to the position of the motif in a peptide library, means to the side or sides of, and does not
25 necessarily mean contiguous or adjacent to.

As used herein, "motif" means a short amino acid sequence, typically comprising 1 to 6 invariant (*i.e.* non-degenerate) amino acids, which occurs in a plurality of peptides or proteins within a genome, and thus is recurring (non-unique). The term includes single amino acid

motifs, such as phosphothreonine, and multiple amino acid motifs, such as comprised in kinase consensus substrate motifs, protein binding motifs, or protein cleavage motifs. Motifs including multiple invariant amino acids may also comprise multiple variant (*i.e.* degenerate) amino acid positions. For example RXRXXT*, the Akt substrate consensus sequence motif, contains 3 invariant amino acids and 3 degenerate amino acids.

As used herein, "motif-specific, context-independent antibody" means an antibody which preferentially recognizes a plurality of peptides or or proteins within a genome that contain the motif for which the antibody is specific; the specificity of the antibody is thus substantially independent of the surrounding protein or peptide context in which the antigenic motif occurs. Motif-specific, context-independent antibodies are thus suitable for genome-wide profiling applications, as the antibodies recognize many, if not most, of proteins within a genome containing the motif. The motif that the antibody preferentially binds may consist of all or part of the "target motif" presented in the immunizing degenerate peptide library, but in either case includes any modified amino acid(s) presented in the target motif.

As used herein, "substrate" means any target molecule, including peptides or proteins, which an enzyme specifically recognizes and acts upon.

The general method by which motif-specific, context-independent antibodies are produced in accordance with the present invention is as follows:

(1) Motif-specific antibodies that specifically recognize many different proteins or peptides containing a desired motif substantially independently of the amino acids flanking the motif may be obtained by constructing (*e.g.* synthesizing), for use as an immunogen, a degenerate

- peptide library comprising (i) a fixed target motif comprising one or more invariant amino acids, and (ii) a plurality of degenerate amino acids flanking the motif. In a preferred embodiment, the target motif includes at least one modified amino acid. For example, in one preferred
- 5 embodiment, the library comprises XXXXXXJ*XXXXXXC where X= all 20 amino acids except cysteine, and the motif, J*,= a modified (*) amino acid (J), for example, phosphothreonine (T*) or acetylated-lysine (K*). The terminal cysteine is used to couple the library to a carrier, thus cysteine is excluded from the degenerate positions to avoid unwanted coupling.
- 10 Other exemplary peptide libraries and motifs are set out in the Examples provided below (e.g. a degenerate library comprising (in place of the J* indicated above) the MAPK consensus substrate motif, PXS*P, is described in Example II). It will be appreciated that the specific target residue(s) of the motif may be unmodified and that a shorter or longer
- 15 library may be generated and less than all of the degenerate amino acids flanking the motif may be varied.

It will be recognized that a peptide library of any length suitable for use as an immunogen to raise anti-peptide antibodies may be advantageously employed in the practice of the invention. The

20 construction and typical range of length of such peptide antigens has been well described. See e.g., ANTIBODIES: A LABORATORY MANUAL, Harlow and Lane Eds., p. 75-76, Cold Spring Harbor Laboratory (1988); Czernik, *supra.* at 266-67. Generally, the length of suitable peptide antigen is only limited by the increasing cost and difficulty of synthesizing

25 ever larger peptides, which do not appreciably improve antigenicity. Thus, in one embodiment, the peptide library of the invention is about 6 to 20 residues long. In another preferred embodiment, the peptide library is about 6 to 14 residues long. In still another preferred embodiment, the peptide library is about 6 to 18 residues long.

The peptide library may be constructed, as in a preferred embodiment, with most, if not all, of the amino acids flanking the motif being highly degenerate; the amino acids at those positions are any of the 19 amino acids (excepting cysteine). However, less highly degenerate libraries may alternatively be constructed to contain bias for or against certain residues at particular flanking amino acid positions, or in which less than all of the flanking amino acids are degenerate. For example, in another preferred embodiment, degenerate amino acids at positions flanking the motif may be any amino acid except cysteine (C) and tryptophan (W). Cysteine is excluded to avoid unwanted coupling, as discussed. Tryptophan is excluded because it is a rare amino acid generally, and rarely occurs in positions surrounding modification sites, such as phosphorylation sites, in proteins. Biasing the flanking amino acid positions against W is also believed to reduce the likelihood of generating any antibody response to W, which is a large and somewhat antigenic residue, thus focusing the antibody response of the host on the desired motif. Tyrosine (Y) may also be excluded for the same reason.

Similarly, certain flanking amino acid positions may be biased for particular residues to increase the antigenicity of the immunizing degenerate peptide library. In some cases, it may be known that a given motif adopts a certain structure, *in vivo*, that is influenced by the presence of particular amino acids surrounding that motif. In such cases, a degenerate peptide library may similarly be constructed with biased flanking residues in an effort to increase the immunogenicity of the fixed motif in the peptide library by introducing structure mimicking that found *in vivo*. For example, if desired, the influence of particular flanking residues may be determined in advance as follows: A library of peptides that contain a single modifiable target residue, such as phosphotyrosine, and varying flanking residues is constructed. The library is reacted with a desired enzyme, such as a particular kinase of interest, to modify the target residue. The modified peptides are then separated from any

unmodified peptides, and batch-sequenced (e.g. by Edman degradation) to examine the abundance of each amino acid at a particular flanking position. An enriched amino acid at a particular flanking position indicates that residue is favored by the enzyme at that flanking position.
 5 (per personnel communication, Lewis Cantley). A degenerated peptide library may then be constructed with bias towards the favored residue(s) one or more flanking positions.

In a preferred embodiment, selected flanking amino acids may be biased in order to achieve about 50% representation of one or two amino
 10 acids at a given degenerate position in the peptide library and about 50% of all other amino acids except C and W represented at that position. This bias is introduced by biasing the coupling mixture for the desired position during synthesis, described below. By way of further example, in a preferred embodiment (see Example XV(b)), context-independent
 15 antibodies specific for the ABL kinase consensus substrate motif (VIY*AXP, where Y* = phosphotyrosine) may be produced by constructing a biased degenerate peptide library comprising CXXAXVIY***A**APFXXX, where bold indicates invariant residues of the motif, X = any amino acid except C and W, and **A** and **F** = flanking positions biased 50% towards
 20 alanine (A) or phenylalanine (F), respectively (the other 50% representing any amino acid except C or W). Where bias towards two or more amino acids at a given degenerate position is desired, the coupling mixture for that position may be prepared with a total bias of 50% to the desired residues, collectively (e.g. total bias of 50% to K and R (in other words
 25 about 25% total bias to each of K and R). It will be recognized that a greater or lesser amount of bias (than 50%) may be advantageously employed. Further examples of such biased peptide libraries are provided in Example XV. It is also anticipated that a mixture of peptides corresponding to approximately 10 known kinase substrates sharing a
 30 common motif may be used as an immunogen to generate antibodies to the common motif that are somewhat context-independent.

The degenerate amino acids in the library, including those within or flanking the motif, may be varied at more than one position simultaneously, or, as in the preferred embodiment, varied at only one surrounding sequence position per degenerate molecule, such that a library is produced which is completely degenerate at every position except the fixed residue(s) of the motif. The peptide library can be synthesized by standard F-Moc solid phase peptide synthesis using an ABI peptide synthesizer and using mixtures of each amino acid during degenerate coupling reactions.

The target motif may consist of a single invariant amino acid (either modified or unmodified), or may comprise multiple amino acids, typically 1 to 6 invariant amino acids, representing a short motif common to a plurality of different peptides or proteins within a genome. The uniqueness of a particular peptide sequence is related to its length, since only one of twenty possible amino acids occurs at each position in the peptide chain. Thus, the probability of a sequence of length (n) occurring at random is $1/20$ raised to the nth power. By way of example, for a particular sequence of length 4 (*i.e.* 4 fixed residues of a motif) there are a total of $(20)^4 = 160,000$ possible amino acid sequence, hence the probability of this specific sequence occurring is $1/160,000 = 6.25 \times 10^{-6}$. For vertebrate genomes encoding approximately 90,000 proteins of an average length of 500 amino acids, there are approximately 30 million different amino acid sequences, so a 4 amino acid sequence motif would occur at random roughly $(90,000 \times 500) \times 6.25 \times 10^{-6} = 300$ times across the entire genome. Similarly, a 5 amino acid motif will occur about 15 times, a 6 amino acid motif will occur around once, and a 7 amino acid motif will occur about 0.05 times across the entire genome.

Thus, from this rough analysis, it is clear that an immunizing peptide sequence should generally be 7 amino acids or greater in length in order to generate an antibody with unique specificity (*i.e.* not reactive

with any other human proteins). For precisely this reason, conventional site-specific peptide immunogens are typically 10-15 amino acids in length, which is small enough to focus the antibody response to the desired region of the protein, yet long enough to ensure multiple overlapping unique epitopes. See, e.g. ANTIBODIES: A LABORATORY MANUAL (1988), *supra*. Epitopes recognized by native immune system antibodies are typically even larger. Conversely, the range of motif length useful for generating conserved motifs across many proteins is between about 1 to 6 amino acids. This short range comports with both statistical prediction, as discussed above, and the observation that most, if not all, kinase consensus motifs reported to date comprise 6 or fewer invariant amino acids. See, e.g. L. Cantley, "Use of Peptide Libraries to Determine the Substrate Specificity of Protein Kinases," in Cell Signaling Technology, Inc.'s Catalogue and Technical Reference (2000-2001), p. 198; Songyang *et al.*, *Current Biology* 4: 973-982 (1994); Songyang *et al.*, *Mol. Cell. Biol.* 16: 6486-6493 (1996); Nishilawa *et al.*, *J. Biol. Chem.* 272: 952-960 (1990); Kemp *et al.*, *Trends Biochem. Sci.* 15: 342-346 (1990); al-Obeid *et al.*, *Biopolymers* 47: 197-223 (1998); Yaffe *et al.*, *Nature Biotech.* 19: 348-353 (2001); PROTEIN PHOSPHORYLATION: A PRACTICAL APPROACH, D. Hardie ed., p. 267, IRL Press (1993).

In preferred embodiments, the target motif comprises at least one modified amino acid, e.g. a phosphorylated amino acid. Alternatively, the motif may comprise multiple modified amino acids (e.g. T*PXXS*P, the GSK consensus substrate motif, comprises two phosphorylated residues). In other embodiments, the motif is an unmodified motif, such as protein cleavage motifs (e.g. caspase cleavage motifs). In certain preferred embodiments, the motif is a single phosphorylated amino acid, a single acetylated amino acid, or a single nitrosylated amino acid. Such preferred motifs include, but are not limited to, a single phosphothreonine, a single phosphoserine, a single phosphotyrosine, a single acetyl-lysine, and a single nitrotyrosine. In other preferred

embodiments, the motif comprises at least two invariant amino acids including at least one phosphotyrosine or phosphothreonine, or comprises at least one phosphoserine but does not consist of phosphoserine-proline. Another preferred motif is phosphothreonine-X-arginine (T*XR). In other preferred embodiments, the motif comprises all or part of a kinase consensus substrate motif or a protein binding motif. Such preferred motifs include, but are not limited to, all of part of MAPK consensus substrate motifs, CDK consensus substrate motifs, PKA consensus substrate motifs, Akt consensus substrate motifs, PKC consensus substrate motifs, ATM consensus substrate motifs, 14-3-3 consensus binding motifs, and bulky-ring/PDK1 consensus docking motifs. In still other preferred embodiments, the motif comprises all or part of PKC Zeta consensus substrate motifs, ABL kinase consensus substrate motifs, CDK5 consensus substrate motifs, insulin receptor consensus substrate motifs, PI3K P85 consensus binding motifs, CaMKII consensus substrate motifs, Src kinase consensus substrate motifs, CDC2/CDK2 consensus substrate motifs, GSK3 kinase consensus substrate motifs, and proline-phosphoserine-proline (PS*P).

The incorporation of modified amino acids at invariant (*i.e.* fixed) positions within the motif in the peptide library should not be limited to phosphorylation or acetylations, as other modified protected amino acids can also be incorporated. For example, motifs comprising one or more amino acids modified with lipids (e.g. farnesylated, isoprenylated) or protected O-linked or N-linked sugars (e.g. glycosylated), methylated, or ribosylated amino acids, or nucleotides, polymers of nucleotides, nucleosides, or amino acids such as ubiquitin, or amino acid analogues may be advantageously employed in the invention.

Alternatively, the incorporation of unmodified amino acids at fixed positions in the peptide library may be selected to mimic conserved motifs, for example zinc fingers or repeating arginine residues.

(2) In order to produce as equal a representation of each non-excluded amino acid as possible at each degenerate position, several rounds of altering the amino acid composition, synthesizing, and peptide sequencing are conducted. Amino acid sequence analysis at several
5 different positions along the peptide is conducted to verify a random amino acid representation at each position and that the random representation is maintained throughout the synthesis. It will be recognized by one of skill in the art that the number of rounds may vary in order to achieve an equal distribution of all amino acids at each position.

10 Alternatively, the representation of particular amino acids at certain degenerate positions in the peptide library may be intentionally biased, as discussed above. For example, in addition to exclusion of cysteine (C) (to avoid unwanted coupling), the rare residue tryptophan (W) may also be generally excluded at each degenerate position. Biasing the
15 degenerate amino acids flanking the motif against W is believed to reduce the likelihood of generating any unwanted antibodies to W, a large, somewhat antigenic residue. Similarly, certain flanking amino acid positions may be biased for particular residues to increase the antigenicity of the immunizing degenerate peptide library, as discussed
20 above. For example, in a preferred embodiment, selected flanking amino acids may be biased in order to achieve about 50% representation of one or two amino acids at a given degenerate position in the peptide library and about 50% of all other amino acids except C and W represented at that position. This bias is introduced at specific degenerate positions by
25 biasing the coupling mixture for the desired position during synthesis, described below. It will be recognized that a greater or lesser amount of bias (than 50%) may be advantageously employed.

(3) The degenerate peptide library is used as an antigen, preferably by covalent coupling to a carrier. In a preferred embodiment,
30 keyhole limpet hemocyanin (KLH) emulsified in Freund's adjuvant is used

as the coupling agent, and the coupled peptide library injected intradermally into a host, such as female New Zealand white rabbits, in order to raise context-independent antibodies specific for a motif consisting of all or part of the target motif, but in either case including the invariant modified residue(s) of the target motif. Antibodies of the invention include those specific for either the target motif itself (in which case the antibodies will not recognize peptides or proteins lacking the entire target motif) or for a part of the target motif (in which case the antibodies will recognize peptides or proteins containing only that part of the target motif, as well as those containing the entire target motif). In the latter case, the motif for which the antibody is specific consists of that part of the target motif that is antigenic. Booster injections may be given in incomplete Freund's adjuvant until an immune response is obtained. Antibody titre is measured by a suitable method, such as ELISA against the motif-specific peptide libraries. Antisera raised in this manner may be used in both crude or purified preparations, as outlined below.

For motifs containing invariant positions that may be two or three specific allowable residues, e.g. bulky ring/PDK1 docking motif ((F/Y)(T*/S*) or (S*/T*)F) and CDC2/CDK2 consensus substrate motif (S*PR(K/R)), a single degenerate peptide library will typically be constructed with a mixture of allowable residues at such positions, and then coupled to the carrier. Alternatively, however, more than one degenerate peptide library, each with only one of the allowable residues at such positions, may first be constructed, coupled to the carrier for immunization, and then the antisera from immunization with each library mixed together. By way of example, a degenerate peptide library comprising the ATM consensus substrate motif, L(T*/S*)Q(D/E), may be constructed as two distinct degenerate libraries, one comprising LT*Q(D/E) and the other LS*Q(D/E), which are used for immunization separately, and the antisera then mixed together (see, e.g., Example XIII).

(4) Antisera from the most promising hosts are purified, for example over protein A, and adsorbed over a J (non-modified motif) peptide library column. In a preferred embodiment, the nonadsorbed fraction (flow through) is then applied to a J* column (modified motif),
5 eluted at suitable pH, dialyzed and tested for J* (modified motif) specificity by a suitable method, such as ELISA using J* and J as antigen.

(5) Antibodies affinity purified in this fashion recognize the J* (modified motif) peptide library but do not react with the J (unmodified
10 motif) library and exhibit a high degree of specificity for J*. These antibodies may be further tested for lack of reactivity against the unmodified form of the target motif (comprising modified amino acid(s), J*), or a J* homologue, utilizing a suitable method, such as ELISA.

(6) Antibodies may be further tested, as in preferred
15 embodiments, by western blotting or another suitable method, using cell extracts prepared from cells treated with and without a selected protein modification enzyme inhibitor, such as protein phosphatase inhibitor okadaic acid. Treatments that increase protein modification will increase the number of antibody reactive proteins as well as the intensity of
20 reactivity. The J* (modified motif)-specific antibodies will react with a relatively small number of proteins from control extracts but will react with a very large number following treatment with the selected inhibitor. The antibodies will show no reactivity with the inactive-non-modified versions
25 of these proteins, demonstrating a high degree of J* specificity and suggesting broad cross-reactivity to many different proteins within a genome that contain the same modified motif.

(7) The degree of context-independence may be more carefully examined, as in preferred embodiments, for example, by ELISA analysis against individual J* (modified motif) peptides that are mixed together or
30 tested individually. Such analysis can indicate if poor reactivity occurs

with certain motifs, such as when J* (modified motif) is followed by proline, for example.

(8) The context-dependence of the J* (modified motif) antibody recognition may be further examined, as in the preferred embodiment, using a immobilized grid of modified-peptide libraries. In addition to a fixed target motif, J*, each different library is synthesized to contain an additional fixed amino acid at different positions relative to J* but with all other positions containing all 20 amino acids except cysteine. Each peptide library is coated, for example, on the bottom of an ELISA well and exposed to the J* antibodies. Motif-specific antibodies that do not react with a particular spot (peptide library) on the grid do not bind when the specified amino acid is present at the specified position. This analysis determines whether or not a particular amino acid at a particular position relative to J* (modified motif) will allow or block binding. Such testing aids in determining whether the antibody is specific for the entire target motif (presented in the degenerate peptide library) or for a part of the target motif, as discussed above. Such testing also confirms the context-independence of the antibody. For example, in a preferred embodiment, the invention provides a context-independent antibody that recognizes a motif consisting of a single phosphothreonine, where the specificity of the antibody is independent of variations in the amino acid residue at the -1 position relative to the motif, as determined by peptide grid (binding) analysis (see Example I).

Alternatively, purified antibodies can be linked to beads, allowed to bind the modified or unmodified library, unbound sequences washed away, and bound sequences recovered and subject to amino acid sequencing to determine the amount of each amino acid present at each position in the library. This information will indicate what amino acids are tolerated at each position.

(9) Monoclonal antibodies may be prepared, as in one form of the preferred embodiment, by coupling the J* (modified motif) degenerate peptide library to a suitable carrier, such as KLH, and injected into a host, such as BalbC mice, in order to raise antibodies specific for the target motif or part of the target motif. The J* peptide-KLH conjugate may be emulsified in Freund's adjuvant and booster injections in incomplete Freund's adjuvant may be carried out every other week until a response is obtained.

(10) Antibody titre is measured by a suitable method, such as ELISA against J* (modified motif) and non-J* peptide libraries. Sera from hosts showing high-titre responses are adsorbed with immobilized non-J* peptide and the nonadsorbed fraction tested by, for example, western blotting.

(11) Spleens from hosts showing J* (modified motif)-specific responses are fused to myeloma cells and hybridoma clones are selected and screened. Supernatants from individual clones are screened first for their ability to bind the J*-peptide library. Positive clones are next screened for their cross-reactivity against the non-J* library. Clones showing the highest degree of J*-specificity are chosen for further analysis as described above in steps (5) through (8).

(12) Overproduction of monoclonal antibodies resulting from step (11) above may be carried out, for example, by harvesting ascites, culturing selected hybridoma clones, or cloning into a host organism, such as *E. coli*.

The motif-specific, context-independent antibodies of the invention produced by this method provide the ability to specifically recognize multiple peptides or proteins within a genome that contain the same motif using a single antibody. In a preferred embodiment, the antibodies of the invention recognize a majority of peptides or proteins containing

the motif within a genome. For example, the antibodies of the invention may be used to identify an unknown substrate of an enzyme. In a preferred embodiment, such antibodies are first generated against the modified form of a motif that is recognized by the enzyme of interest, for example, a consensus site. These antibodies are then used to screen a sample for the presence of other, unknown substrates which contain the same modified motif. This method enables the rapid detection of important new substrates in a variety of cascades which involve conserved substrate motifs. For example, antibodies that selectively recognize a wide variety of proteins only when phosphorylated at the MAPK consensus phosphorylation site would greatly facilitate the detection of new MAP kinase targets. The highly context-independent antibodies of the invention enable such genome-wide profiling, as they recognize many of, if not most, peptides or proteins containing the same short motif. MAP kinase could be overexpressed in cell culture, activated by growth factors, and target substrate proteins identified by western blotting using antibodies that selectively recognize the phosphorylated substrate proteins (Stukenberg *et al.*, *Curr. Biol.* 7:338-348 (1997). Alternatively, MAPK could be used to phosphorylate cDNA expression libraries *in vitro* and MAPK consensus-site antibodies used to identify cDNA clones expressing MAPK phosphorylated substrates (Funkunaga and Hunter, *EMBO* 16(8):1921-1933 (1997). Similarly, the method may be employed to identify new substrates containing specific unmodified motifs, e.g. protein cleavage motifs.

Similarly, motif-specific, context-independent antibodies of the instant invention may be used to identify an enzyme which modifies a known substrate motif. Such antibodies, whether specific for modified (e.g. phosphorylated) or unmodified (e.g. zinc finger) motifs, can be used to detect whether a certain enzyme of interest has modified a substrate which contains that motif. This method allows for the rapid detection of important new proteins which act on known classes of substrates

containing contain conserved motifs, for the example MAPK consensus site. In a preferred embodiment, the antibody recognizes the modified form of the motif, and an enzyme sample is reacted with known substrate containing the unmodified form of the motif, and the antibody then used
5 to screen whether any substrate has been modified by the enzyme. Alternatively, the method may be employed to identify enzymes that act on unmodified motifs, such as protein cleavage motifs.

The motif-specific, context-independent antibodies of the invention may also be used *in vitro* as reagents in high-throughput assays, such as
10 drug screens, to detect the enzymatic modification of certain substrates containing a conserved motif in a cell or tissue. For example, antibodies specific for a certain phosphorylated motif enable the rapid detection of inhibitors of the enzyme that act at that motif. In the case of a drug screen, a single motif-specific antibody can be used to assay the activity
15 of a wide range of enzymes acting at many diverse sequence motifs. Phosphotyrosine antibodies are currently employed in high throughput kinase assays to screen for selective, high affinity tyrosine kinase inhibitors. Compounds or drugs that block enzyme activity are detected by their ability to inhibit kinase activity as determined by a reduction of
20 phosphotyrosine antibody binding to phosphorylated substrate. Similar assays can be set up to screen for pharmaceutically useful compounds using antibodies produced as described above for phosphoserine, phosphothreonine, or antibodies detecting other protein modifications. In a preferred embodiment, the antibody recognizes the modified form of a
25 common motif, and is used to screen an extract of a cell or tissue treated with a drug to profile drug-induced changes in the level or post-translational modification of proteins in the extract that contain the modified motif.

Antibody based detection of protein kinase activity has several
30 advantages over radioactive assays for use in automated high throughput

kinase assays. First, radioactive assays are difficult to automate because they employ transfer of ^{32}P gamma-labeled ATP to a peptide substrate. The phosphopeptide is then separated from labeled ATP using phosphocellulose filters and several washing steps, and finally, phosphorylation is quantitated by liquid scintillation methods. Together these steps are time consuming and difficult to automate. Antibody detection allows a wide variety of ELISA-type assays that are well suited for automation and high throughput screens.

Second, radioactive assays require low levels of ATP to insure high levels of ^{32}P incorporation for maximal sensitivity. Low levels of ATP in the kinase assay bias the search for inhibitors towards compounds that compete with ATP binding in the protein kinase catalytic cleft. Such screens consistently yield competitive inhibitors at the ATP binding site which due to the highly conserved nature of this binding site results in inhibitors with poor selectivity.

Current high-throughput kinase assays typically utilize biotinylated peptide substrates immobilized on the bottom of a 96 or 386 well plate that is subsequently incubated together with the desired protein kinase, ATP, and the appropriate kinase buffer. Kinase activity is measured using a fluorescently labeled phosphospecific-antibody that reacts only with the phosphorylated peptide substrate. These assays come in two formats homogeneous (not involving wash steps) and heterogeneous (involving wash steps). Homogeneous fluorescent assays typically utilize lanthanide-labelled phosphoantibody binding to a phosphorylated peptide substrate that has linked to it an energy acceptor, for example allophycocyanin. Binding of the phosphoantibody the phosphorylated peptide substrate brings the two fluorophores close enough together to allow fluorescence resonance energy transfer to occur shifting the frequency of the emitted signal, indicating the presence of a biomolecular complex. Different compounds are added to each well and

the ability of the compound to inhibit substrate phosphorylation is determined by inhibition of fluorescence energy transfer. This format is similar to the scintillation proximity assay commonly used in radioactive assays. Other homogeneous assays involve the use of fluorescence polarization to measure the binding of phosphoantibody to phosphorylated substrate.

The key feature in the homogeneous assays are the limited number of steps and the ease in automation. A large variety of heterogeneous kinase assays based upon ELISA formats are also currently in use. These assays typically utilizing fluorescently labeled phosphoantibodies binding phosphorylated peptide substrates that are immobilized in 96 or 386 well formats. In this case wash steps are required to separate bound from unbound antibody. Fluorescently labeled antibody retained in the well is then detected using time resolved fluorescence.

The motifs used to generate antibodies for such modification screening assays may be either modified or unmodified substrate motifs. Antibodies generated against unmodified motifs will not bind if the substrate has been subsequently modified by an enzyme. Similarly, antibodies generated against modified motifs can detect increases in modified substrate concentrations owing to enzymatic activity.

Similar approaches may be applied to study a variety of other enzymatic modifications, and are not limited to the protein kinase or acetyltransferase activities discussed below. For example, the approach could be used to generate antibodies that recognize many other types of protein modification, including, but not limited to, the addition of sugars, methyl groups, carboxyl groups, the addition of various lipids, or the addition of nucleotides, or polymers of nucleotides, nucleosides, or amino acids such as ubiquitin.

Likewise, such motif-specific, context-independent antibodies may be used on a genome-wide scale to simultaneously profile large and diverse protein populations (e.g. in a cell, tissue, or fluid) which contain conserved motifs. A specific two or three amino acid binding site, for example consecutive arginine residues, should appear (based upon a random distribution of amino acids) once every 400 or 8000 residues, respectively, (equating to approximately once per protein, or once every 20 proteins, respectively, (assuming the average protein is 400 amino acids)). Thus, an antibody that specifically recognizes such a motif in a manner that is substantially independent of the context in which it occurs allows for the rapid screening of a great number of proteins or peptides containing the motif. For example, there at least 14 known human proteins containing the AKT consensus substrate motif. See Yaffe *et al.*, *supra* at p. 350 (Table 1). A context-independent antibody specific for all or part of this motif thus allows for the single-antibody detection of these many motif-containing AKT substrates, as well as the identification of other unknown AKT substrates containing the same consensus motif.

Phosphorylation specific antibodies allow genome wide profiling of changes in phosphorylation of proteins (e.g. in a cell, tissue, or fluid) as a result of drug treatment or the overexpression of specific genes/proteins as a result of such treatment. Such antibodies also facilitate the profiling of expression of specific proteins in sequenced genomes.

For example, suppose that a drug is developed which inhibits the cell-cycle dependent protein kinase cdc2. The drug has been shown to inhibit cdk2 with high affinity, but the specificity of the compound needs to be further tested to examine whether other protein kinases are inhibited and if so, which ones.

As an early step in this process cell lines may be treated with the drug and the effects on total cell protein phosphorylation monitored using

a panel of motif-specific and general phosphoantibodies to examine the nature of the phospho-substrates inhibited by the compound or lead drug.

Total protein from cell extracts prepared from control or drug treated cells may be fractionated using, for example, 2-dimentional gels (isoelectric focusing in the first dimension and standard SDS-polyacrylamide molecular weight fractionation in the second dimension), transferred to nitrocellulose membranes, and analyzed by western blotting using, in this hypothetical case, kinase consensus site-specific phosphoantibodies. Alternatively, genome-wide modification may be profiled by analyzing complex mixtures of peptides in cellular digests, as described in USSN 60/299,893 (Rush *et al.*, filed June 2, 2001, assigned to Cell Signaling Technology, Inc.).

In this case, global analysis of total cell proteins using a cdc2 consensus site specific antibody would provide information regarding the ability of the drug to block phosphorylation at all potential cdc2 site substrates. The pattern of inhibition at other non-cdc2 substrates (*i.e.* the degree of specificity) could also be examined using antibodies to different kinase consensus sites, or using antibodies to phosphotyrosine to determine whether the inhibitor also acts to block tyrosine kinases.

Currently, for mammalian cells, the the identity of the majority of protein "spots" visualized on 2-D gels are unknown. However, as all human genes are identified and sequenced and the corresponding proteins characterized and "spots" identified, analysis by protein profiling in accordance with the present invention will become even more powerfully informative. The identity of the proteins inhibited will not only confirm the drug specificity but the identity of additional "nonspecific" proteins inhibited will also suggest possible side effects. Identical analysis can be carried out in simpler, completely sequenced organisms, such as yeast where many of the protein "spots" on 2-D gels have already been identified.

The Examples presented below are only intended as specific preferred embodiments of the present invention and are not intended to limit the scope of the invention except as provided in the claims herein. The present invention encompasses modifications and variations of the methods taught herein which would be obvious to one of ordinary skill in the art.

The references cited above and below are herein incorporated by reference.

EXAMPLE 1

10 **Context-Independent Phosphothreonine Antibodies**

Synthesis of peptide library antigens:

Phospho-specific antibodies that react with any protein containing phosphorylated threonine residues, i.e that bind phosphothreonine independently of the surrounding amino acids, were obtained by synthesizing a highly degenerate peptide library XXXXXXThr*XXXXXXC where X= all 20 amino acids except cysteine and Thr* = phosphothreonine.

The phosphothreonine peptide library was synthesized by standard F-Moc solid phase peptide synthesis using an ABI peptide synthesizer and using mixtures of each amino acid during degenerate coupling reactions. Degenerate peptides were synthesized using an ABI model 433A peptide synthesizer, using FastMoc chemistry (Fields *et al.*, *Pept. Res.* 4:95-101 (1991), hereby incorporated by reference herein) at a scale of 0.085 mmol. Fmoc/NMP chemistry utilizing HBTU amino acid activation (Dourtoglou *et al.*, *Synthesis* 1984: 572-574 (1984), Knorr *et al.*, *Tetra. Let.* 30:1927-1930 (1989), Knorr *et al.*, in Peptides 1988 37-129 (1989), Walter de Grueter & Co, all hereby incorporated by reference herein) was employed for all cycles. Preloaded Fmoc-Cys(Trt) HMP (p-

hydroxymethylphenoxyethyl) polystyrene resin functionalized at 0.5 mmol/g was used for each degenerate pool of peptides. Peptides were synthesized using single coupling during each cycle, although coupling times were extended at each position containing a phosphorylated amino acid. The final Fmoc was removed during synthesis. Utilization of preloaded HMP resin along with final Fmoc group removal yields peptides having both free amino and carboxy termini after cleavage and deprotection.

In order to produce as equal a representation of each amino acid as possible at each degenerate position several rounds of altering the amino acid composition, synthesizing, and peptide sequencing were conducted. The desired peptide pools were to contain an equimolar mix of 19 amino acids (all standard amino acids except Cys) at each degenerate site. Because the rate of reactivity of each protected amino acid differs, simply mixing equimolar amounts (each at approximately 5.26% of total) does not result in a population of peptides that is equimolar at each position. In order to maximize degeneracy at each residue, peptide synthesis was first done using equimolar "mixes" at each position. Phenylthiocarbamyl-amino acid analysis was performed therefore allowing assessment of relative amino acid content at each position. Based on amino acid analysis the molar amounts of each amino acid in the "mix" were adjusted to compensate for different reaction rates, in order to ensure equal representation of each amino acid at each degenerate position. Several rounds of peptide synthesis followed by amino acid analysis were necessary to optimize the amino acid mix, which resulted in a totally degenerate peptide. The optimized amino acid mix arrived at was as follows: G (4.6%); A (5.6%); V (3.3%); L (2.5%); I (4.25%); S (4.4%); T (8.4%); F (2.25%); Y (6.0%); W (6.8%); M (2.9%); P (2.5%); D (5.8%); N (9.5%); E (6.2%); Q (9.4%); K (6.1%); R (6.4%); H (3.5%).

Cleavage of the degenerate peptides from the resin along with removal of side chain protecting groups occurs simultaneously upon treatment with TFA. The cleavage mixture (Perkin Elmer, Emeryville, CA (1995)) consists of the following: 0.75g phenol, 0.125 ml methyl sulfide, 5 0.25 ml 1,2-ethanedithiol, 0.5 ml milliQ H₂O, 0.5 ml thioanisol, 10 ml TFA. The entire mixture was added to the peptide resin (approx. 300 mg). The resin was flushed with nitrogen and gently stirred at room temperature for 3 hours. The resin was then filtered allowing the peptide to be precipitated into cold (0°C) methyl-t-butyl ether. The ether fraction was 10 centrifuged allowing collection of the precipitate. The peptide precipitate was vacuum dried, analyzed by mass spectroscopy, and HPLC purified.

A sample of the peptide was dissolved in acetonitrile/water (50:50, v/v) and analyzed on a Perceptive Biosystems (Framingham, MA) MALDI-TOF mass spectrometer using 2,4,6-trihydroxyacetophenone plus 15 ammonium citrate as the matrix. As expected, the peptide mixture did not show a homogeneous product. MALDI-TOF analysis demonstrated that the peptide pool was degenerate, showing an average mass and the expected statistically normal curve of peptide mass.

Peptides were purified using a Waters HPLC system consisting of 20 a Lambda-Max Model 481 Multiwavelength detector, 500 series pumps, and Automated gradient controller. A Vydac semi-preparative C18 column was used for reverse-phase purification. A 60 min. linear gradient, 10%-100% B, was used at a flow rate of 2 ml/minute. Buffer A consisted of 0.1% TFA/H₂O (v/v) while buffer B consisted of 0.1% 25 TFA/60% CH₃CN/40% H₂O (v/v/v). Detection was at 214 nm.

Because the peptide pool was degenerate (as demonstrated by mass spectroscopy) HPLC purification was not expected to yield a homogeneous product. Base-line separation of peptide mixtures was not achieved by this method and it was only intended as a crude 30 purification/desalting step. Mass spectroscopy was performed and all

fractions whose mass was within the theoretical range were pooled and lyophilized.

Amino acid sequence analysis at several different positions along the peptide indicated a random amino acid representation at each
5 position and that the random representation was maintained throughout the synthesis. The results indicated the production of highly diverse peptide libraries that would serve as suitable antigens.

Production of Rabbit polyclonal antibodies:

All peptides synthesized contained C-terminal cysteine residues
10 allowing conjugation to the carrier protein (KLH) using the heterobifunctional cross-linking reagent m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS). The conjugation procedure used was as described by the manufacturer (Pierce), although the amount of peptide coupled to KLH was increased to 10 mg in order to provide increased
15 material for immunization and boosting of animals. Scale-up required use of a larger desalting column (Bio-Rad 10 DG (Cambridge, MA)) to remove the excess MBS after reaction to N-termini and the ϵ -amino group of KLH Lysine residues.

The phosphothreonine peptide library was covalently coupled to
20 keyhole limpet hemocyanin (KLH) (250 μ grams), emulsified in Freund's adjuvant and injected intradermally into female New Zealand white rabbits. Booster injections (200 μ grams) in incomplete Freund's adjuvant were carried out every other week until a response was obtained. Rabbit sera was screened at three week intervals for the presence of
25 phosphopeptide specific immunoreactivity by ELISA using both the phosphothreonine and nonphosphothreonine peptide libraries. When the titre of antibody against phosphopeptide reached 10^5 , rabbits were put on a production bleed schedule with bleeds collected every two weeks.

When 40 ml of high titre serum were obtained, purification of phosphospecific antibodies was initiated, as described below.

Antisera from the most promising rabbit was purified over protein A and passed over a nonphospho Thr/Ser peptide library column. The
5 nonadsorbed fraction (flow through) was applied to a phosphothreonine column, eluted at low pH, dialyzed and tested for phosphospecificity by ELISA using phospho- and nonphosphopeptides. Antibodies affinity-purified in this fashion recognized the phosphorylated threonine peptide library but did not react with the nonphosphothreonine/serine library,
10 indicating a high degree of specificity for phosphothreonine (see Figure 1a). ELISA results also indicated that the antibodies also reacted specifically with a mixture of 18 different phosphothreonine peptides but showed no reactivity with any of the corresponding nonphosphopeptides (Figure 1b). The antibodies also exhibited a strict preference for
15 phosphothreonine, showing no reactivity with a mixture of 38 different phosphoserine peptides (Figure 1b) or peptides containing phosphotyrosine.

We next tested the antibodies by western blotting using cell extracts prepared from cells treated with and without the protein
20 phosphatase inhibitor okadaic acid. As shown in Figure 1c the phosphothreonine antibodies react with a relatively small number of proteins from control extracts but react with a very large number following treatment with okadaic acid (see the smear of high Mol Wt. reactive proteins in Figure 1c, lane 2). The antibodies also reacted specifically
25 with the active forms of MAPK (ERK1) and MKK3 only when phosphorylated at threonine residues at their respective activation loops. The antibodies showed no reactivity with the inactive-nonphosphorylated versions of these proteins (Figure 1c, lanes 3-6). These results demonstrate a high degree of phosphothreonine specificity and suggest

broad cross-reactivity to many different threonine-phosphorylated proteins and peptides.

To examine more carefully the degree of context-independence, ELISA analysis was conducted against individual threonine phosphorylated peptides that were mixed together in the previous experiment. As shown in Figure 1a, the phosphothreonine antibody reacts well with all phosphopeptides except those where phosphothreonine is immediately followed by proline, for example the c-Myc and APP1 phosphopeptides (Figure 2b). These results indicate that purified rabbit antibodies reacted in a phosphospecific manner with a wide variety of phosphothreonine but react only poorly with phosphopeptides where the phosphorylated threonine is followed by proline.

The context-dependence of phosphothreonine antibody recognition was further examined using an immobilized grid of phosphopeptide libraries. In addition to a fixed phosphothreonine, each different library was synthesized to contain an additional fixed amino acid at the -4, -3, -2, -1, +1, +2, +3 positions relative to phosphothreonine but with all other positions containing all 20 amino acids except cysteine. Each peptide library was coated on the bottom of an ELISA well and exposed to the phosphothreonine antibodies. Antibodies that do not react with a particular spot (peptide library) on the grid do not bind when the specified amino acid is present at the specified position. This analysis determines whether or not a particular amino acid at a particular position relative to phosphothreonine will allow or block binding (Figure 1d).

Results confirmed that the phosphothreonine antibodies tolerated all amino acids in the -1, -2, -3, -4, and +2, +3 position, and bound equally well to every amino acid except proline at the +1 position (see Figure 1d, first row). The reactivity as defined by this binding profile indicates that

the antibodies will bind all phosphothreonine containing sequences except those followed immediately in -1 position by proline. Further analysis using a variety of specific phosphothreonine containing peptides confirmed these results.

- 5 Phosphothreonine specific antibodies from several other rabbits immunized with the same peptide library antigens were further purified and characterized. Antibodies purified from sera obtained from two other rabbits also produced broadly cross-reacting phosphothreonine antibodies as determined by ELISA. One rabbit produced antibodies that
- 10 react equally well with peptides containing proline following the phosphothreonine. Taken together, these results demonstrate the broad context-independence of the phosphothreonine response obtained when combinatorial peptide libraries are used as immunogens.

EXAMPLE II

15 **Protein Kinase Consensus Site-Specific Phosphoantibodies**

MAPK-consensus recognition sites: PXS*P

- A peptide library of the preferred site for MAPK phosphorylation PXS*P was synthesized (Figure 2a) substantially as described in Example I. In addition to an equimolar mix of phosphoserine and
- 20 threonine, amino acids at two other positions were also fixed; proline at -2 and proline at +1. This library was coupled to KLH and injected into rabbits as described for phosphothreonine. IgG from the most promising rabbit was protein A purified and passed over a nonphospho-Thr/Ser peptide library column. The nonadsorbed fraction (flow through) was
- 25 applied to a phospho-PXS*P column, eluted at low pH, dialyzed and tested for phosphospecificity by ELISA using phospho- and nonphosphopeptides.

Antibodies affinity purified in this fashion reacted strongly with the phosphorylated PXS*P peptide library but did not react with the nonphosphothreonine/serine library (see Figure 2a). ELISA results also indicated that the antibodies also reacted specifically with a mixture of 18 different phosphothreonine peptides but showed no reactivity with any of the corresponding nonphosphopeptides (Figure 2a). In addition to being phosphospecific, the antibodies exhibited a preference for proline at the -2 and +1 positions and showed no reactivity with phosphorylated peptides that lack proline at this position (Figure 2a). The antibodies reacted strongly with the RB and cdk4 phosphopeptides but showed no reactivity with the MKK3, PKCalpha, or p70S6 phosphopeptides that lack proline at the +1 position (Figure 2a). These antibodies do react with some peptides lacking proline at -2, for example the cdk4 phosphopeptide, suggesting that proline at this position is not absolutely necessary.

PXS*P antibodies were further tested by western blotting using cell extracts prepared from cells treated with and without the protein phosphatase inhibitor okadaic acid. Binding of the PXS*P antibodies to cell extracts from RS 4;11 cells was strongly enhanced following treatment with okadaic acid (smear of high Mol Wt. proteins in Figure 2b, lane 2). The antibodies also reacted specifically with ATF-2 phosphorylated *in vitro* with MAP kinase but not the nonphosphorylated form of this protein (Figure 2b, lanes 3 and 4), demonstrating a high degree of phospho-specificity and broad cross-reactivity to many different phosphorylated proteins and peptides.

The specificity of PXS*P antibody recognition was also examined using an immobilized grid of phosphopeptide libraries. As described above, in addition to a fixed phosphothreonine or phosphoserine, each different library was synthesized to contain an additional fixed amino acid at the -1, +1, +2 positions relative to phosphothreonine but with all other positions containing all 20 amino acids except cysteine.

The PXS*P antibody reacted weakly with peptide libraries where proline was fixed at the -1 position and reacted strongly with libraries where proline was fixed at both the -2 and +1 positions. The reactivity as defined by this binding profile indicates that the PXS*P antibodies
5 strongly bind only sequences containing the PXS*P motif, as expected, but that the antisera still contain some residual reactivity to S*P (as a result of impurities), which could be removed by further purification using immobilized S*P peptide library.

EXAMPLE III

10 Protein Kinase Consensus Site-Specific Phosphoantibodies

14-3-3 binding site: RSXS*XP

Antibodies that identify 14-3-3 targets were obtained by synthesizing a peptide library: XXXXRSXS*XPXXXXC where S* is phosphoserine and X represents any amino acid and C is cysteine. The
15 above 14-3-3 phosphopeptide library was synthesized by standard F-Moc solid phase peptide synthesis using an ABI peptide synthesizer and mixtures of each amino acid except cysteine during degenerate coupling reactions, as discussed in Example I.

The 14-3-3 phosphopeptide library was coupled to KLH and
20 injected into rabbits as described above for phosphothreonine and PXS*P. Antisera from the most promising rabbit was purified over protein A and adsorbed over a nonphospho-14-3-3 peptide library column. The flow-through of this column was applied to a phospho-14-3-3 column eluted at low pH, dialyzed and tested for phosphospecificity by ELISA
25 using phospho- and nonphospho-14-3-3 peptide libraries. These affinity purified phospho-14-3-3 antibodies recognized the phosphorylated 14-3-3 peptide library but not the nonphospho-14-3-3 library, indicating a high degree of specificity for phospho-14-3-3 (see Figure 3a). The antibodies

also reacted strongly with several different peptides containing the 14-3-3 motif including; phospho-Bad-Ser136, cdc25-Ser216, and more weakly with phospho-Bad-Ser112 which contains a slight variant motif. The antibodies showed no reactivity with the corresponding nonphospho-peptides (Figure 3a) or with many other phosphopeptides that did not contain the motif.

Phospho-14-3-3 antibodies were further tested by western blotting using cell extracts prepared from cells transfected with a GST-Bad fusion protein and treated with and without the phorbol ester TPA. The antibodies reacted with a small number of proteins from control extracts (see Figure 3b). Bad was detected in extracts prepared from transfected cells but not control cells. Since the basal level of Bad phosphorylation is high it was difficult to see increased phosphorylation with TPA, although TPA did induce the phosphorylation of several higher molecular weight proteins (arrow in Figure 3b). These results indicate that the phospho-14-3-3 antibodies can detect phosphorylated Bad and other TPA stimulated phospho-proteins.

ELISA analysis against the previously described grid of serine/threonine phosphorylated peptide libraries was also conducted. As expected, the phospho-14-3-3 antibodies have an absolute requirement for proline at the +2 position.

A second, monoclonal, motif-specific, context-independent antibody that recognizes the 14-3-3 motif #1 was also produced, as described in Example XIV(a), below.

EXAMPLE IV**Production of Mouse Monoclonal Antibodies: CDK Consensus Phosphorylation Site PXT*PXR:**

The PXT*/S*PXR sequence represents a consensus
5 phosphorylation site for many of the cell cycle-dependent proteins
kinases (cdks). Antibodies that recognize this phosphorylated motif
would be useful to identify new cdk substrates important in controlling cell
cycle progression. The PXT*/S*PXR peptide library shown in Figure 4a
was coupled to KLH and injected into Balb/c mice. The phosphopeptide-
10 KLH conjugate (50 µgrams) emulsified in Freund's adjuvant was injected
IP. Booster injections (12.5 to 25 µgrams) in incomplete Freund's
adjuvant were carried out every three weeks until a response was
obtained. Antibody titre was measured by ELISA against the immunized
phosphopeptide library. Sera from mice showing high-titre responses
15 were adsorbed with immobilized nonphospho Thr/Ser peptide and the
nonadsorbed fraction tested by western blotting (data not shown).

Splenocytes from a mouse showing phosphospecific responses
were fused to myeloma X63Ag8.635 cells (Kearney *et al.*, *J. Immunol.*
123:1548-1550 (1979)) and approximately 1,100 hybridoma clones were
20 selected and screened. Supernatants from individual clones were
screened first for their ability to bind the immunized phosphopeptide
library and next for their cross-reactivity against the non-phosphopeptide
library. Two different clones showing the highest degree of phospho-
specificity were chosen for further analysis. The specificity of clones 6B8
25 and 5A9 were further characterized using the phosphopeptide libraries
and phosphopeptides shown in Figure 4a. Both clones reacted
specifically with phosphothreonine containing libraries and individual
peptides but did not significantly react with phosphoserine containing
peptides, indicating that phosphothreonine selective clones had been
30 identified. Both clones reacted strongly with peptide libraries where

proline is fixed in the -2 and +1 positions relative to phosphothreonine. Reactivity against T*P and PXT*P libraries does not indicate relaxed specificity since one of 400 and one of 20 peptides in the respective libraries will have the appropriate amino acids at the fixed positions. Both clones reacted strongly with a single RB phosphothreonine peptide containing each of the fixed positions present in the immunized library but did not react significantly with the corresponding nonphosphopeptide.

Western analysis shows that okadaic acid treatment of cultured cells dramatically increases the reactivity with both clones 6B8 and 5A9 (Figure 4b). Clone 6B8 is also shown to detect cdc2 phosphorylated RB by western blotting (Figure 4b) but does not react with nonphosphorylated RB protein. Clone 5A9 was deposited in accordance with the terms and conditions of the Budapest Treaty on September 4, 1998 with the American Type Culture Collection under ATCC Accession No. HB12563.

Subsequent testing indicated the specificity of this antibody requires T*P, a part of the target motif which includes the modified residue. Hence, this motif-specific, context-independent antibody is capable of recognizing multiple proteins that contain a T*P motif, including where this motif is presented within the CDK consensus substrate motif (PXT*PXR).

EXAMPLE V

Acetylated-Lysine Specific Antibodies

Antibodies specifically reactive against acetylated lysine but not reactive against non-acetylated lysine were obtained by synthesizing the following acetylated lysine peptide library: XXXXXXK*XXXXXXC where K* is acetylated and X represents any amino acid except cysteine and C is cysteine. The acetylated lysine peptide library was synthesized as

described previously by standard F-Moc solid phase peptide synthesis using commercially available fully protected acetylated lysine.

The peptide library was coupled to KLH and injected into rabbits. The K*-peptide-KLH conjugate (250 µgrams) was used as immunogen as described for the other phosphopeptide libraries. Antisera from the most promising rabbit were purified over protein A and adsorbed over a non-acetylated lysine peptide library column. The flow through of this column was applied to an acetylated lysine column, eluted at low pH, dialyzed and tested for phosphospecificity by ELISA.

Acetylated-lysine antibodies, affinity purified as described above, recognized the acetylated lysine peptide library but not the non-acetylated library, indicating a high degree of specificity for acetylated lysine as measured by ELISA. The antibodies also reacted specifically with as little as 0.5 ng of acetylated bovine serum albumin (BSA) but showed no reactivity with up to 10 µgrams of nonacetylated BSA (see Figure 5a).

The antibodies were further examined by western blotting using cell extracts prepared from cells treated with and without anisomycin. The antibodies react with a number of different proteins present in the C6-cell extracts (Figure 5b). In panels b and c, antibodies were preincubated with 1 µgram of nonacetylated peptide library (Figure 5b) or 1 µgram of acetylated peptide library (Figure 5c). Preincubation with nonacetylated peptide library had little effect on antibody reactivity with acetylated control protein or bands visualized in the cell extract (Figure 5c, lanes 5-8). However, preincubation of the antibodies with the acetylated lysine peptide library completely blocked antibody binding to control acetylated BSA as well as binding to many proteins present in the cell extract (Figure 5d, lanes 9-12). These results demonstrate a high degree of specificity for acetylated lysine and indicate that the antibodies recognize a broad spectrum of different sized proteins that contain acetylated lysine in a

variety of surrounding sequence contexts (compare Figure 5c and d, lanes 1, 2).

EXAMPLE VI

Phosphoantibody to the Substrate Consensus Sequence for Akt: RXRXXT*

5 The Akt protein kinase is an important regulator of cell survival and insulin signaling, but very few of its *in vivo* targets have been identified. Studies with synthetic peptide substrates of Akt (D.R. Alessi *et al.* FEBS Lett. 399:333-338 (1996)) as well as the analysis of known Akt
10 phosphorylation sites on GSK-3 (T.F. Franke *et al.* Cell 88:435-437 (1997)), Bad (M. Pap *et al.* J. Biol. Chem. 273:19929-19932 (1998); Datta *et al.* Cell 91:231-241 (1997)), FKHR Brunet *et al.* Cell 96:857-868 (1999)), and Caspase-9 (M.H. Cardone *et al.* Science 282:1318-1321 (1998)) indicate that Akt phosphorylates its substrates only at a serine or
15 threonine in a conserved motif characterized by arginine at positions -5 and -3.

To study and discover new Akt targets, an antibody was developed that specifically recognizes the phosphorylated form of the Akt substrate consensus sequence RXRXXT*. This antibody was raised against the
20 following synthetic peptide antigen, where X represents a position in the peptide synthesis where a mixture of all twenty amino acids (excluding cysteine) were used, and Thr* represents phospho-threonine: Cys-X-X-X-Arg-X-Arg-X-X-Thr*-X-X-X-X (SEQ ID NO: 45). The synthetic phospho-peptide was conjugated KLH (keyhole limpet hemocyanin) and injected into
25 rabbits. Test bleeds were collected and characterized by ELISA on phospho and non-phospho versions of the peptide antigen.

Once rabbits started to show high phospho-specific titers, 40ml production bleeds were obtained. Bleeds were dialyzed overnight in

0.025M NaAcetate, 0.01M NaCl pH=5.2 at 4°C, then spun at 11,200 rpm at 4°C for 30min to precipitate serum lipids. Serum supernatant was then purified by Protein A chromatography on a Pharmacia ÄKTA FPLC to isolate the IgG antibody fraction. Affinity chromatography is then performed using peptide coupled to SulfoLink resin from Pierce (#20401; coupling directions according to manufacturer). Phospho-Akt Substrate Antibody was found to be already highly phospho-specific as crude serum, so that a subtraction step on a column containing the non-phospho peptide was not necessary and the elution from the Protein A column could be used directly for affinity chromatography on a phospho-peptide-containing column. Protein A eluate was incubated with phospho-peptide resin by rotation in a sealed column at room temperature for one hour. Column was then drained, washed twice with PBS, and eluted with 0.1M Glycine, pH 2.7 and pooled fractions neutralized with 1M Tris-HCl, pH 9.5 (~1-2% of fraction volume). The eluted phospho-specific antibody was then dialyzed overnight in PBS at 4°C.

The resulting antibody is highly specific for peptides which contain phospho-threonine/serine preceded by arginine at positions -5 and -3 (Figure 6). Some cross-reactivity is observed for peptides which contain arginine at positions -3 and -2. (Figure 6) also shows that this antibody is highly phospho-specific and recognizes these motifs only when phosphorylated (signal to noise ratios were determined as a ratio of reactivity with the phospho-peptide to reactivity with the corresponding non-phospho-peptide). This antibody does not recognize other phospho-threonine/serine containing motifs. (Figure 7) indicates that in mammalian cells there are many phosphoproteins recognized by this antibody.

A second context-independent antibody that recognizes the Akt substrate consensus motif, RXRXXT* was produced by synthesizing the following biased degenerate peptide library: CXXRXRX**T**X**T***FGXXX (SEQ ID NO: 88), where T* is phosphothreonine and bold indicates

invariant motif residues, X represents any amino acid excluding cysteine (C) and tryptophan (W), and underlined residues, T, F, and G = degenerate positions at which the representation of amino acids is biased to 50% of the underlined residue (threonine (T), phenylalanine (F), or glycine (G), respectively) and 50% of all other amino acids excluding cysteine and tryptophan. Bias towards these particular residues at the given degenerate positions was chosen based upon the prior determination of flanking residues (in peptide substrates) favored by Akt kinase, substantially as described above.

10 The degenerate peptide library was synthesized by standard F-Moc solid phase peptide synthesis using an ABI peptide synthesizer and amino acid mixtures (further excluding tryptophan (W)), substantially as described in Example 1. However, for the biased degenerate positions indicated, the mixtures of amino acids used during degenerate coupling
15 reactions were as follows: 50% of the coupling mixture comprised the biased flanking residue (T, F, or G, respectively) and the other 50% comprised a mixture of all amino acids, substantially as described in Example 1, excluding C and W.

 This biased degenerate peptide library was coupled to KLH and
20 injected into rabbits as described above in Example 1. Antisera from the most promising rabbit was purified over protein A and adsorbed over an unmodified (nonphospho) Akt consensus substrate motif peptide library column. The flow-through of this column was applied to a modified (phospho) – Akt consensus motif column eluted at low pH, dialyzed and
25 tested for phosphospecificity. Initial results indicate these antibodies are phospho-specific (data not shown), and it is anticipated that further specificity testing by ELISA, as outlined in Example 1, will indicate these motif-specific, context-independent antibodies (as with those produced with an unbiased peptide library) are highly specific for the phosphorylated form
30 of all or part of the Akt consensus substrate motif, and will specifically

recognize a plurality of different proteins within a genome that contain this motif.

EXAMPLE VII

Phosphoantibody to the Substrate Consensus Sequence for PKA: RRXT*

5

cAMP-dependent Protein Kinase A (PKA) is an important kinase for regulating a striking number of physiologic processes, including intermediary metabolism, cellular proliferation and neuronal signaling, by altering basic patterns of gene expression (M. Montminy Annual Rev. Biochem. 66:807-822 (1997)). Studies with synthetic peptide substrates have established a consensus phosphorylation site for PKA, namely serine or threonine with arginine at the -2 and -3 positions (Songyang *et al.*, *supra.*).

To identify and study new *in vivo* substrates of PKA, an antibody was developed that specifically recognizes the phosphorylated form of the PKA substrate consensus sequence RRXT*. The following synthetic phospho-peptide peptide antigen was used, where X represents a position in the peptide synthesis where a mixture of all twenty amino acids (except cysteine) were used, and Thr* represents phospho-threonine: Cys-X-X-X-X-X-Arg-Arg-X-Thr*X-X-X-X (SEQ ID NO: 46). The synthetic phospho-peptide was conjugated KLH (keyhole limpet hemocyanin) and injected into rabbits. Test bleeds were collected and characterized by ELISA on phospho and non-phospho versions of the peptide antigen.

Once rabbits started to show high phospho-specific titers, 40ml production bleeds were obtained. Bleeds were dialyzed overnight in 0.025M NaAcetate, 0.01M NaCl pH=5.2 at 4°C, then spun at 11,200rpm at 4°C for 30min to precipitate serum lipids. Serum supernatant was then purified by Protein A chromatography on a Pharmacia (Piscataway, NJ)

ÄKTA FPLC to isolate the IgG antibody fraction. Affinity chromatography was then performed using peptide coupled to SulfoLink resin from Pierce (#20401; coupling directions according to manufacturer). Both phospho-peptide-containing resin and the corresponding non-phospho-peptide resin were prepared. Protein A eluate was first incubated with non-phospho-peptide resin by rotation in a sealed column at room temperature for one hour, in order to remove antibodies reactive with the non-phospho version of the protein antigen. This resin was then drained and the flow-through then incubated with phospho-peptide resin. This column was drained, washed twice with PBS, phospho-specific antibody eluted with 0.1M Glycine, pH 2.7 and pooled fractions neutralized with 1M Tris-HCl, pH 9.5 (~1-2% of fraction volume). The eluted phospho-specific antibody was then dialyzed overnight in PBS at 4°C.

Figure 8 shows that the resulting antibody is highly specific for peptides or proteins containing phospho-threonine with arginine at the -3 position. The antibody also recognizes some proteins containing phospho-serine with arginine at the -2 and -3 position. It does not recognize the non-phosphorylated version of these motifs (as shown by the signal to noise ratios in Figure 8 which were determined as a ratio of reactivity with the phospho-peptide to reactivity with the corresponding non-phospho-peptide); nor does the antibody recognize other phospho-serine/threonine containing motifs. Figure 9 indicates that in mammalian cells there are many phosphoproteins recognized by this antibody, while Figure 10 shows that this antibody specifically detects many PKA protein substrates in a cell but will not recognize substrates of the ERK2 or CDC2 kinases, which have different substrate specificities.

EXAMPLE VIII

Phosphoantibody to the Substrate Consensus Sequence for Bulky Ring-Directed Kinases/PDK1 Docking Motif: [F/Y][T/S]* or [S/T]*F

Some important classes of protein kinases are regulated by phosphorylation of a specific serine or threonine flanked by either phenylalanine or tyrosine. For example, Akt, which plays a central role in regulating cell survival, is activated by phosphorylation at Ser473, a site flanked by phenylalanine and tyrosine (D.R. Alessi *et al.* EMBO J. 15:6541-6551 (1996)). RSK1 (Ser381) and the PKC's also contain this consensus site, phosphorylation of which is required for their activity (K.N. Dalby *et al.* J. Biol. Chem. 273:1496-1505 (1998); L.M. Keranen *et al.* Curr. Biol. 5:1395-1403 (1995)).

To help study signaling pathways regulated by phosphorylation at these key regulatory sites we developed an antibody that detects phosphoserine and phospho-threonine only when preceded by tyrosine, tryptophan or phenylalanine or when followed by phenylalanine (the "bulky-ring kinase"/PDK1 consensus docking motif. This antibody was raised against the following synthetic peptide antigen, where X represents a position in the peptide synthesis where a mixture of all twenty amino acids (except cysteine) were used, and Ser* or Thr* represents phospho-serine or phospho-threonine: X-X-X-X-F-X-X-F-[S*/T*]-[F/Y]-X-X-X-X-C (SEQ ID NO: 47). This synthetic phospho-peptide was conjugated to KLH and injected into rabbits. Test bleeds were collected and characterized by ELISA on phospho and non-phospho versions of the peptide antigen.

Once rabbits started to show high phospho-specific titers, 40ml production bleeds were obtained. Bleeds were dialyzed overnight in 0.025M NaAcetate, 0.01M NaCl pH=5.2 at 4°C, then spun at 11,200 rpm at 4°C for 30min to precipitate serum lipids. Serum supernatant was then purified by Protein A chromatography on a Pharmacia (Piscataway, NJ)

ÄKTA FPLC to isolate the IgG antibody fraction. Affinity chromatography was then performed using peptide coupled to SulfoLink resin from Pierce (#20401; coupling directions according to manufacturer). Both phospho-peptide-containing resin and the corresponding non-phospho-peptide resin were prepared. Two rounds of subtractive purification were performed using the non-phospho-peptide resin: Protein A eluate was incubated with non-phospho-peptide resin by rotation in a sealed column at room temperature for one hour, in order to remove antibodies reactive with the non-phospho version of the protein antigen. The column was drained and the flow-through (containing the desired antibody) incubated with fresh non-phospho-peptide resin. The flow-through from this second subtractive step was finally positively purified by incubation with phospho-peptide resin. After the phospho-peptide column was drained and washed twice with PBS, phospho-specific antibody (bound to the resin) was eluted with 0.1M Glycine, pH 2.7 and pooled fractions were neutralized with 1M Tris-HCl, pH 9.5 (~1-2% of fraction volume). The eluted phospho-specific antibody was then dialyzed overnight in PBS at 4°C.

The resulting antibody is highly specific for phosphorylated [F/Y][T/S]- or [S/T]F- containing peptides (Figure 11). It does not recognize non-phosphorylated [F/Y][T/S] or [S/T]F motifs or other phospho-serine/threonine containing proteins and peptides (signal to noise ratios were determined as a ratio of reactivity with the phospho-peptide to reactivity with the corresponding non-phospho-peptide). This antibody does not recognize other phospho-threonine/serine containing motifs. Figure 12 indicates that in mammalian cells there are many phosphoproteins recognized by this antibody.

A second, monoclonal, motif-specific, context-independent antibody that recognizes the bulky ring/PDK1 consensus docking motif was also produced, as described in Example XIV(b), below.

EXAMPLE IX

Context-Independent Antibody Specific for the PKC Consensus Substrate Motif: (K/R)S*(F/L/V)(K/R)(K/R)

Although Protein Kinase C (PKC) family members are involved in a number of cellular signal transduction processes including secretion, gene expression, proliferation and muscle contraction (K. Nishikawa *et al.* (1997) J. Biol. Chem. 272:952–960; R.B. Pearson and B.E. Kemp (1991) Methods Enzymol. 200:62–81), many of its substrates remain unidentified. Isozymes of PKC are subdivided into conventional PKCs (cPKC), novel PKCs (nPKC) and atypical PKCs (aPKC). PKC α , β I, β II, and γ isoforms belong to cPKC (K. Nishikawa *et al.* (1997) J. Biol. Chem. 272:952–960). When activated, cPKC isozymes phosphorylate substrates containing serine or threonine, with arginine or lysine at the –3, –2 and +2 position, and hydrophobic amino acids at position +1 (K. Nishikawa *et al.* (1997) J. Biol. Chem. 272:952–960; R.B. Pearson and B.E. Kemp (1991) Methods Enzymol. 200:62–81; T. Obata *et al.* (2000) J. Biol. Chem. 275:36108–36115).

To assay PKC activity and identify and characterize new *in vivo* substrates of PKC, a context-independent antibody was produced that specifically recognizes the phosphorylated form of the PKC substrate consensus sequence (K/R)(K/R)S*(F/L/V)(K/R)(K/R). The following synthetic degenerate peptide library was constructed, substantially as described in Example 1, where invariant residues of the motif are indicated in bold (K/R means lysine or arginine must be present at that position; F/L/V means phenylalanine, leucine, or valine must be present), X represents any amino acid except W, C, or Y (further excluded in this example because of its size), and S* is phosphoserine:

CXXX(K/R)(K/R)S*(F/L/V)(K/R)(K/R)XXX (SEQ ID NO: 89) The synthetic phospho-peptide was conjugated to KLH (keyhole limpet hemocyanin) and injected into rabbits, substantially as described in Example 1.

Once rabbits started to show high phospho-specific titers, 40ml production bleeds were obtained. Bleeds were dialyzed overnight in 0.025M NaAcetate, 0.01M NaCl pH=5.2 at 4°C, then spun at 11,200 rpm at 4°C for 30min to precipitate serum lipids. Serum supernatant was then
5 purified by Protein A chromatography on a Pharmacia (Piscataway, NJ) ÄKTA FPLC to isolate the IgG antibody fraction. Affinity chromatography was then performed using peptides coupled to SulfoLink resin from Pierce (#20401; coupling directions according to manufacturer). Both phospho-peptide-containing resin and the corresponding non-phospho-peptide resin were prepared. Two rounds of subtractive purification were
10 performed using the non-phospho-peptide resin: Protein A eluate was incubated with non-phospho-peptide resin by rotation in a sealed column at room temperature for one hour, in order to remove antibodies reactive with the non-phospho version of the protein antigen. The column was
15 drained and the flow-through (containing the desired antibody) incubated with fresh non-phospho-peptide resin. The flow-through from this second subtractive step was finally positively purified by incubation with phospho-peptide resin. After the phospho-peptide column was drained and washed twice with PBS, phospho-specific antibody (bound to the resin)
20 was eluted with 0.1M Glycine, pH 2.7 and pooled fractions were neutralized with 1M Tris-HCl, pH 9.5 (~1-2% of fraction volume). The eluted phospho-specific antibody was then dialyzed overnight in PBS at 4°C.

The resulting antibody was characterized by ELISA on phospho
25 and non-phospho versions of the peptide antigen. By ELISA Phospho-(Ser) PKC Substrate Antibody detects phosphorylated serine residue in a number of peptide sequences with Arg or Lys at the -2 and +2 position and a hydrophobic residue at the +1 position relative to phospho-serine (Figure 13). Western blot analysis with the antibody shows that it
30 recognizes a plurality of different proteins within a genome in response to TPA treatment (known to stimulate PKC signaling; Figure 14). Figure 15

resolves these TPA-stimulated epitopes better by performing 2-dimensional electrophoresis prior to western blotting.

EXAMPLE X

5 **Context-Independent Antibody Specific for a Single Nitrotyrosine Motif**

10 Nitric oxide (NO) is implicated in carcinogenesis (B.G. Bentz *et al.* (2000) Head Neck 22:64–70), chronic infection, inflammation (M. Jaiswal *et al.* (2000) Cancer Res. 60:184–190) and neurodegeneration (R. Olivenza *et al.* (2000) J. Neurochem. 74:785–791). High levels of both
15 superoxide and nitric oxide in these tissues interact to form peroxynitrite, a potent oxidant that can modify Tyr residues in proteins to form 3-nitrotyrosine (L.A. MacMillan-Crow *et al.* (1996) Proc. Natl. Acad. Sci. USA 93:11853–11858). Tyrosine nitration of mitochondrial manganese superoxide dismutase results in loss of enzymatic activity (L.A. MacMillan-Crow *et al.* (1996) Proc. Natl. Acad. Sci. USA 93:11853–11858). The nitration of p53 at Tyr residues abolishes its capacity for binding to its DNA consensus sequence (L. Chazotte-Aubert *et al.* (2000) Biochem. Biophys. Res. Commun. 267:609-613).

20 To study known nitro-tyrosine-containing proteins and identify new ones, a context-independent antibody was developed that specifically recognizes a motif consisting of a single nitrated tyrosine (YNO₂). The following synthetic degenerate peptide library was constructed, substantially as described in Example 1, where bold indicates the invariant residue of the motif, X represents a degenerate flanking position
25 where any amino acid except cysteine may occur, and YNO₂ represents nitrated tyrosine: CXXXXXX(YNO₂)XXXXXXX. The degenerate peptide library was conjugated to KLH (keyhole limpet hemocyanin) and injected into rabbits, substantially as described in Example 1.

Once rabbits started to show high nitro-tyrosine-specific titers, 40ml production bleeds were obtained. Bleeds were dialyzed overnight in 0.025M NaAcetate, 0.01M NaCl pH=5.2 at 4°C, then spun at 11,200 rpm at 4°C for 30min to precipitate serum lipids. Serum supernatant was then
5 purified by Protein A chromatography on a Pharmacia (Piscataway, NJ) ÄKTA FPLC to isolate the IgG antibody fraction. Affinity chromatography was then performed using peptides coupled to SulfoLink resin from Pierce (#20401; coupling directions according to manufacturer). Both nitro-tyrosine-peptide resin and the corresponding non-nitrotyrosine
10 peptide resin were prepared. Two rounds of subtractive purification were performed using the non-nitro-tyrosine peptide resin: Protein A eluate was incubated with non-nitro-tyrosine peptide resin by rotation in a sealed column at room temperature for one hour, in order to remove antibodies reactive with the non-nitrated version of the protein antigen. The column
15 was drained and the flow-through (containing the desired antibody) incubated with fresh non-nitro-tyrosine peptide resin. The flow-through from this second subtractive step was finally positively purified by incubation with nitro-tyrosine peptide resin. After the nitro-tyrosine peptide column was drained and washed twice with PBS, nitro-tyrosine-
20 specific antibody (bound to the resin) was eluted with 0.1M Glycine, pH 2.7 and pooled fractions were neutralized with 1M Tris-HCl, pH 9.5 (~1-2% of fraction volume). The eluted nitro-tyrosine-specific antibody was then dialyzed overnight in PBS at 4°C.

The resulting antibody was characterized by ELISA on nitrated and
25 non-nitrated versions of the peptide antigen. The nitrotyrosine polyclonal antibody is highly specific for nitrated tyrosine, in a manner substantially independent of surrounding amino acid sequence. It does not recognize unmodified tyrosine residues or tyrosine modified by phosphorylation. Reactivity with *in vivo* epitopes is shown in Figure 16, where extracts of
30 cells treated with Peroxynitrite show enhanced western blot signal, indicating reactivity with a plurality of different nitrotyrosine-containing

proteins, but cells treated with degraded peroxynitrite (which will not cause tyrosine nitration) or with pervanadate (which causes tyrosine phosphorylation but not nitration) do not show increased immunoreactivity by western blot, as expected. Figure 17 shows a similar result, increased immunoreactivity in peroxynitrite-treated cells, this time using the antibody for immunocytochemistry.

EXAMPLE XI

Context-Independent Antibody Specific for the Phosphothreonine-X-Arginine Motif

Some important signaling proteins can be regulated by phosphorylation at a specific threonine followed by arginine or lysine at the +2 position. For example, conventional PKC isozymes phosphorylate substrates containing serine or threonine with arginine or lysine at the -3, -2 and +2 position (K. Nishikawa et al. (1997) J. Biol. Chem. 272:952-960; R.B. Pearson and B.E. Kemp (1991) Methods Enzymol. 200:62-81). c-Raf, a mitogen-activated protein kinase and the main effector recruited by GTP-bound Ras, is phosphorylated at threonine 481 and 491 followed by lysine at the +2 position (B. Zhang, and K. Guan (2000) EMBO J. 19:5429-5439). APP1 and ERF also contain a phosphorylated threonine followed by arginine or lysine at the +2 position. Phosphorylation of these sites is important for the regulation of the activity of these proteins.

To assay the activity of basic-directed kinases, study their substrates and identify new substrates, a context-independent antibody was developed that specifically recognizes the phosphothreonine-X-arginine (T*XR) motif. The following degenerate peptide library was constructed, substantially as described in Example 1, where bold indicates invariant residues of the motif, X represents degenerate flanking positions in the peptide synthesis where a mixture of all 20 amino acids excluding cysteine was used, and T* represents phosphothreonine:

CXXXXXXT*XRXXXX. The degenerate peptide library was conjugated to KLH (keyhole limpet hemocyanin) and injected into rabbits.

Once rabbits started to show high phospho-specific titers, 40ml production bleeds were obtained. Bleeds were dialyzed overnight in 0.025M NaAcetate, 0.01M NaCl pH=5.2 at 4°C, then spun at 11,200 rpm at 4°C for 30min to precipitate serum lipids. Serum supernatant was then purified by Protein A chromatography on a Pharmacia (Piscataway, NJ) ÄKTA FPLC to isolate the IgG antibody fraction. Affinity chromatography was then performed using peptide coupled to SulfoLink resin from Pierce (#20401; coupling directions according to manufacturer). Both phospho-peptide-containing resin and the corresponding non-phospho-peptide resin were prepared. Two rounds of subtractive purification were performed using the non-phospho-peptide resin: Protein A eluate was incubated with non-phospho-peptide resin by rotation in a sealed column at room temperature for one hour, in order to remove antibodies reactive with the non-phospho version of the protein antigen. The column was drained and the flow-through (containing the desired antibody) incubated with fresh non-phospho-peptide resin. The flow-through from this second subtractive step was finally positively purified by incubation with phospho-peptide resin. After the phospho-peptide column was drained and washed twice with PBS, phospho-specific antibody (bound to the resin) was eluted with 0.1M Glycine, pH 2.7 and pooled fractions were neutralized with 1M Tris-HCl, pH 9.5 (~1-2% of fraction volume). The eluted phospho-specific antibody was then dialyzed overnight in PBS at 4°C.

The resulting antibody was characterized by ELISA on phospho and non-phospho versions of the peptide antigen (Figure 18). Phosphothreonine-X-Arginine antibody detects phosphorylated threonine followed by arginine or lysine at the +2 position, though its reactivity for lysine at the +2 position is somewhat lower than for arginine. The

antibody does not cross-react with non-phosphothreonine or with phospho-serine in the same motif. It recognizes phospho-threonine in the FFT*R motif in PKC beta II, but does not recognize phospho-threonine in other motifs that lack lysine or arginine at +2. Calyculin A treatment of cells in Figure 19, inhibiting phosphatases and thereby enhancing protein phosphorylation, shows the antibody recognizes a plurality of different proteins containing the same motif. Figure 20 shows immunostaining of a breast cancer tissue section using this antibody, indicating the relevance of this epitope in signaling in tumor tissue.

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EXAMPLE XII

Context-Independent Antibody Specific for 14-3-3 Binding Motif #2: RX(F/Y)XS*

The 14-3-3 proteins are a highly conserved family of proteins involved in the regulation of cell survival, apoptosis, proliferation and checkpoint control (A. Aitken (1995) Trends Biochem. Sci. 20: 95-97; J. Zha et al. (1996) Cell 87:619-628; H. Piwnica-Worms (1999) Nature 401:535-537; G. Tzivion et al. (1998) Nature 394:88-92; Xing, H. (2000) EMBO J. 19:349-358). Binding of 14-3-3 is phospho-serine-dependant (Yaffe et al., *supra.*). Two different phospho-serine containing motifs are found using a degenerate phospho-serine-oriented peptide library technique, RSXS*XP and RX(Y/F)XS*XP (Yaffe et al., *supra.*). Motif 2 (RX(Y/F)XS*XP) (SEQ ID NO: 90) is found in critical regulatory proteins including cdc25a, cdc25b, PKCg, IRS-1 and BCR (Yaffe et al., *supra.*).

To identify and characterize proteins that contain motif #2 and potentially bind 14-3-3, a context-independent antibody was developed that specifically recognizes the 14-3-3 motif #2, RX(F/Y)XS*X, without the requirement for proline +2 to the phosphoserine. The following degenerate peptide library was constructed, substantially as described in Example 1, where bold indicates invariant residues of the motif, X

represents any amino acid except cysteine, and S* represents phosphoserine: XXXRX(F/Y)XS*XPXXXC (SEQ ID NO: 91). The degenerate peptide library was conjugated to KLH (keyhole limpet hemocyanin) and injected into rabbits, as described in Example 1.

- 5 Once rabbits started to show high phospho-specific titers, 40ml production bleeds were obtained. Bleeds were dialyzed overnight in 0.025M NaAcetate, 0.01M NaCl pH=5.2 at 4°C, then spun at 11,200 rpm at 4°C for 30min to precipitate serum lipids. Serum supernatant was then purified by Protein A chromatography on a Pharmacia (Piscataway, NJ)
- 10 ÄKTA FPLC to isolate the IgG antibody fraction. Affinity chromatography was then performed using peptide coupled to SulfoLink resin from Pierce (#20401; coupling directions according to manufacturer). Both phospho-peptide-containing resin and the corresponding non-phospho-peptide resin were prepared. Two rounds of subtractive purification were
- 15 performed using the non-phospho-peptide resin: Protein A eluate was incubated with non-phospho-peptide resin by rotation in a sealed column at room temperature for one hour, in order to remove antibodies reactive with the non-phospho version of the protein antigen. The column was drained and the flow-through (containing the desired antibody) incubated
- 20 with fresh non-phospho-peptide resin. The flow-through from this second subtractive step was finally positively purified by incubation with phospho-peptide resin. After the phospho-peptide column was drained and washed twice with PBS, phospho-specific antibody (bound to the resin) was eluted with 0.1M Glycine, pH 2.7 and pooled fractions were
- 25 neutralized with 1M Tris-HCl, pH 9.5 (~1-2% of fraction volume). The eluted phospho-specific antibody was then dialyzed overnight in PBS at 4°C.

 The resulting Arg-X-(Tyr/Phe)-X-Ser* motif-specific, context-independent antibody recognizes the 14-3-3 binding motif #2, although

30 the recognition does not absolutely require proline in the +2 position.

Thus, the antibody recognizes a large part of the target motif including the phosphorylated residue. The antibody provides a powerful new tool for the discovery and characterization of potential 14-3-3 binding motif #2—containing proteins or other proteins with the RX(F/Y)XS* motif. Calyculin

5 A treatment of cells in Figure 21, inhibiting phosphatases and thereby enhancing protein phosphorylation, shows that this antibody is substantially context-independent, recognizing a plurality of different proteins, *in vivo*, that contain the same motif.

EXAMPLE XIII

10 **Context-Independent Antibody Specific for ATM Consensus Substrate Motif: L(T*/S*)Q(D/E)**

ATM (ataxia telangiectasia mutated kinase) and ATR (ataxia telangiectasia and rad3-related kinase) are related kinases that regulate cell-cycle checkpoints and DNA repair (M.B. Kastan and D.S. Lim (2000)

15 Nature Reviews Mol. Cell. Biol. 1:179–186.) The identified substrates for ATM are p53, p95/NBS1, MDM2, Chk2, BRCA1, CtlP, 4E-BP1 and Chk1 (M.B. Kastan and D.S. Lim (2000) Nature Reviews Mol. Cell. Biol. 1:179–186; H. Zhao and H. Piwnicka-Worms (2001) Mol. Cell. Biol. 21:4129–4139). The essential consensus sequence for the substrates of

20 ATM/ATR is (S/T)Q. Hydrophobic amino acids and negatively charged amino acids immediately N-terminal to serine or threonine are positive determinants and positively charged amino acids in the region are negative determinants. The complex phenotype of AT cells suggests that it must have other cellular substrates as well (S.T. Kim et al. (1999)

25 J. Biol. Chem. 274:37538–37543).

To assay ATM activity and identify and characterize new ATM and ATR substrates, a context-independent antibody was produced that specifically recognizes a plurality of different proteins within a genome containing the phosphorylated substrate motif, (S*/T*)Q. The following

two degenerate peptide libraries were synthesized, substantially as described in Example 1, where bold indicates invariant residues of the motif, where X represents a degenerate flanking position where a synthesis mixture of all 20 amino acids excluding cysteine was used, and where S* is phosphoserine and T* is phosphothreonine:
CXXXXXXLT***Q(D/E)**XXXXX and CXXXXXXLS***Q(D/E)**XXXXX (SEQ ID NOs: 92 and 93). These two degenerate peptide libraries were separately conjugated to KLH (keyhole limpet hemocyanin) and injected into rabbits, as described in Example 1, and the antisera resulting from each immunization were then mixed.

Once rabbits started to show high phospho-specific titers, 40ml production bleeds were obtained. Bleeds were dialyzed overnight in 0.025M NaAcetate, 0.01M NaCl pH=5.2 at 4°C, then spun at 11,200 rpm at 4°C for 30min to precipitate serum lipids. Serum supernatant was then purified by Protein A chromatography on a Pharmacia (Piscataway, NJ) ÄKTA FPLC to isolate the IgG antibody fraction. Affinity chromatography was then performed using peptide coupled to SulfoLink resin from Pierce (#20401; coupling directions according to manufacturer). Both phospho-peptide-containing resin and the corresponding non-phospho-peptide resin were prepared. Two rounds of subtractive purification were performed using the non-phospho-peptide resin: Protein A eluate was incubated with non-phospho-peptide resin by rotation in a sealed column at room temperature for one hour, in order to remove antibodies reactive with the non-phospho version of the protein antigen. The column was drained and the flow-through (containing the desired antibody) incubated with fresh non-phospho-peptide resin. The flow-through from this second subtractive step was finally positively purified by incubation with phospho-peptide resin. After the phospho-peptide column was drained and washed twice with PBS, phospho-specific antibody (bound to the resin) was eluted with 0.1M Glycine, pH 2.7 and pooled fractions were neutralized with 1M Tris-HCl, pH 9.5 (~1-2% of fraction volume). The

eluted phospho-specific antibody was then dialyzed overnight in PBS at 4°C.

The resulting antibody was characterized by ELISA on phospho and non-phospho versions of the peptide antigens (Figure 22). Phospho- (Ser/Thr) ATM substrate consensus antibody detects various peptide sequences that contain phosphorylated serine or threonine when followed by glutamine at the +1 position and preceded by hydrophobic amino acids at the -1 position. In Figure 23, extracts of COS cells treated with UV and probed by western blot with this antibody show enhanced protein phosphorylation in cells treated with UV, relative to untreated cells, indicating a plurality of different proteins contain this motif. Similarly, Figure 24 shows by western blot that this antibody recognizes the motif in a known ATM substrate, Chk2; Chk2 was immunoprecipitated from cells treated with UV (known to stimulate ATM signaling), and by western blotting shows increased immunoreactivity relative to untreated cells.

EXAMPLE XIV

Monoclonal Motif-Specific, Context-Independent Antibodies Specific for Protein Binding Motifs

A. 14-3-3 Binding Motif #1

As described in Example XII above, the 14-3-3 proteins are a highly conserved family of proteins involved in the regulation of cell survival, apoptosis, proliferation and checkpoint control. Two different phospho-Ser-containing motifs are found within nearly all known 14-3-3 binding proteins. Motif #1 (RSXS*XP) is found in critical regulatory proteins including Bad, cdc25c, FKHRL1, PKC and c-Raf (H. Xing (2000) EMBO J. 19:349-358; Yaffe *et al.*, *supra.*). The production of a polyclonal motif-specific, context-independent antibody that recognizes a

plurality of different proteins within a genome that contain the 14-3-3 motif #1 was described in Example III.

A monoclonal context-independent antibody specific for part of this motif was produced by constructing, substantially as described in
 5 Example 1, the following degenerate peptide library (somewhat similar to that described in Example III): CXXXRSXS*XPXXX (SEQ ID NO: 94), where S* is phosphoserine, bold indicates invariant residues of the motif, and X is any amino acid except cysteine. The degenerate peptide library was conjugated to KLH (keyhole limpet hemocyanin) and injected into
 10 mice, as described in Example 1. Splenocytes from mice showing phosphospecific responses were selected and fused to produce hybridomas substantially as described in Example IV.

Clones were screened, as described, and clone 4E2 was selected for further analysis. The resulting antibody produced by clone 4E2 was
 15 characterized by ELISA on phospho and non-phospho versions of the peptide antigen (Figure 25). Phospho- 14-3-3 binding motif monoclonal antibody detects phosphorylated serine in various peptides in the context of arginine at the -3 position, and proline at the +2 position. Calyculin A treatment of cells in Figure 26, inhibiting phosphatases and thereby
 20 enhancing protein phosphorylation, shows that this antibody recognizes a plurality of different proteins containing this motif.

Clone 4E2 was deposited in accordance with the terms and conditions of the Budapest Treaty on October 31, 2001 with the American Type Culture Collection (ATCC) and received Patent Accession No.
 25 PTA-3823.

B. Bulky-ring/PDK1 Consensus Docking Motif

As described in Example VIII above, many critical protein kinases can be regulated by phosphorylation at a specific serine or threonine in a

hydrophobic motif. RSK2, p70 S6 K and certain PKC isoforms also contain a similar consensus phosphorylation motif, FXXF(S*/T*)(F/Y). Phosphorylation of these motifs is required for binding to 3-phosphoinositide-dependent kinase 1 (PDK1) (M. Frodin *et al.* (2000) EMBO J. 19:2924–2934; A. Balendran *et al.* (1999) J. Biol. Chem. 274:37400–37406; A. Balendran *et al.* (2000) J. Biol. Chem. 275:20806–20813). The production of a polyclonal motif-specific, context-independent antibody that recognizes a plurality of different proteins within a genome that contain the bulky-ring/PDK1 consensus docking motif was described in Example VIII.

A monoclonal context-independent antibody specific for this motif was produced by constructing, substantially as described in Example 1, the following degenerate peptide library (identical to that described in Example VIII): XXXXFXXF(S*/T*)(F/Y)XXXXC, where S* is phosphoserine and T* is phosphothreonine, bold indicates invariant residues of the motif, and X is any amino acid except cysteine. The degenerate peptide library was conjugated to KLH (keyhole limpet hemocyanin) and injected into mice, as described in Example 1. Splenocytes from mice showing phosphospecific responses were selected and fused to produce hybridomas substantially as described in Example IV.

Clones were screened, as described, and clone 18A2 was selected for further analysis. The resulting antibody produced by clone 18A2 was characterized by ELISA on phospho and non-phospho versions of the peptide antigen (Figure 27). This phospho-PDK1 docking motif monoclonal antibody detects phosphorylated serine or threonine that is surrounded by tyrosine or phenylalanine at the –1 and +1 position and phenylalanine or lysine at the –4 position. Calyculin A treatment of cells in Figure 28, inhibiting phosphatases and thereby enhancing protein phosphorylation, shows that this motif-specific, context-independent

antibody recognizes a plurality of different proteins within a genome that containing this motif. Figure 29 shows that this antibody works for immunoprecipitation, namely that it will recognize the phospho-PDK1 docking motif within Akt following Akt phosphorylation in response to PDGF treatment (a treatment known to stimulate Akt signaling).

Clone 18A2 was deposited in accordance with the terms and conditions of the Budapest Treaty on October 31, 2001 with the American Type Culture Collection (ATCC) and received Patent Accession No. PTA-3824.

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EXAMPLE XV

Context-Independent Antibodies Specific for Other Motifs

A. PKC Zeta Consensus Substrate Motif

PKC Zeta (protein kinase C zeta) is a kinase that phosphorylates its substrates at a short consensus motif, FXRXXT*FF (SEQ ID NO: 95). Context-independent antibodies specific for the PKC Zeta consensus substrate motif, FXRXXT*FF, were produced by synthesizing the following biased degenerate peptide library: CXRRFKRQGT*FFYFFXX (SEQ ID NO: 96), where T* is phosphothreonine and bold indicates invariant motif residues, X represents any amino acid excluding cysteine (C) and tryptophan, and underlined residues, R, K, Q, G, Y, and F = degenerate positions at which the representation of amino acids is biased to 50% of the underlined residue (arginine (R), lysine (K), glutamine (Q), glycine (G), tyrosine (Y), or phenylalanine (F), respectively) and 50% of all other amino acids excluding cysteine and tryptophan. Bias towards these particular residues at the given degenerate positions was chosen based upon the prior determination of flanking residues (in peptide substrates) favored by PKC Zeta kinase, substantially as described above.

The degenerate peptide library was synthesized by standard F-Moc solid phase peptide synthesis using an ABI peptide synthesizer and amino acid mixtures (further excluding tryptophan (W)), substantially as described in Example 1. However, for the biased degenerate positions indicated, the mixtures of amino acids used during degenerate coupling reactions were as follows: 50% of the coupling mixture comprised the biased flanking residue (R, K, Q, G, Y, or F, respectively) and the other 50% comprised a mixture of all amino acids, substantially as described in Example 1, excluding C and W.

The PKC Zeta motif degenerate peptide library was coupled to KLH and injected into rabbits as described above in Example 1. Antisera from the most promising rabbit was purified over protein A and adsorbed over an unmodified (nonphospho) PKC Zeta consensus motif peptide library column. The flow-through of this column was applied to a modified (phospho) – PKC Zeta consensus motif column eluted at low pH, dialyzed and tested for phosphospecificity. Initial results indicate these antibodies are phospho-specific (data not shown), and it is anticipated that further specificity testing by ELISA, as outlined in Example 1, will indicate these motif-specific, context-independent antibodies are highly specific for the phosphorylated form of the PKC Zeta consensus substrate motif, and will specifically recognize a plurality of different proteins within a genome that contain this motif.

B. ABL Consensus Substrate Motif

ABL (Abelson protein tyrosine kinase) is a kinase that phosphorylates its substrates at a short consensus motif, VIY*AXP. Context-independent antibodies specific for the ABL kinase consensus substrate motif, VIY*AXP (SEQ ID NO: 97), were produced by synthesizing the following biased degenerate peptide library: CXXAXVIY*AAPEXXX (SEQ ID NO: 98), where Y* is phosphotyrosine

and bold indicates invariant motif residues, X represents any amino acid excluding cysteine (C) and tryptophan (W), and underlined residues, A, and F = degenerate positions at which the representation of amino acids is biased to 50% of the underlined residue (alanine (A) or phenylalanine (F), respectively) and 50% of all other amino acids excluding cysteine and tryptophan. Bias towards these particular residues at the given degenerate positions was chosen based upon the prior determination of flanking residues (in peptide substrates) favored by ABL kinase, substantially as described above.

10 The degenerate peptide library was synthesized by standard F-Moc solid phase peptide synthesis using an ABI peptide synthesizer and amino acid mixtures (further excluding tryptophan (W)), substantially as described in Example I. However, for the biased degenerate positions indicated, the mixtures of amino acids used during degenerate coupling
15 reactions were as follows: 50% of the coupling mixture comprised the biased flanking residue (A or F, respectively) and the other 50% comprised a mixture of all amino acids, substantially as described in Example 1, excluding C and W.

20 The ABL kinase consensus motif degenerate peptide library was coupled to KLH and injected into rabbits as described above in Example 1. Antisera from the most promising rabbit was purified over protein A and adsorbed over an unmodified (nonphospho) ABL kinase consensus motif peptide library column. The flow-through of this column was applied to a modified (phospho) – ABL kinase consensus motif column eluted at
25 low pH, dialyzed and tested for phosphospecificity. Initial results indicate these antibodies are phospho-specific (data not shown), and it is anticipated that further specificity testing by ELISA, as outlined in Example 1, will indicate these motif-specific, context-independent antibodies are highly specific for the all or part of the phosphorylated form

of the ABL consensus substrate motif, and will specifically recognize a plurality of different proteins within a genome that contain this motif.

C. CDK5 Consensus Substrate Motif

CDK5 (cyclin dependent kinase 5) is a kinase that phosphorylates its substrates at a short consensus motif, KXXXT*PXHR (SEQ ID NO: 99). Context-independent antibodies specific for the CDK5 consensus substrate motif, KXXXT*PXHR, were produced by synthesizing the following biased degenerate peptide library: CXX**KHHKT*PK**HRXX (SEQ ID NO: 100), where T* is phosphothreonine and bold indicates invariant motif residues, X represents any amino acid excluding cysteine (C) and tryptophan, and underlined residues, H, and K = degenerate positions at which the representation of amino acids is biased to 50% of the underlined residue (histidine (H) or lysine (K), respectively) and 50% of all other amino acids excluding cysteine and tryptophan. Bias towards these particular residues at the given degenerate positions was chosen based upon the prior determination of flanking residues (in peptide substrates) favored by CDK5 kinase, substantially as described above.

The degenerate peptide library was synthesized by standard F-Moc solid phase peptide synthesis using an ABI peptide synthesizer and amino acid mixtures (further excluding tryptophan (W)), substantially as described in Example I. However, for the biased degenerate positions indicated, the mixtures of amino acids used during degenerate coupling reactions were as follows: 50% of the coupling mixture comprised the biased flanking residue (H or K, respectively) and the other 50% comprised a mixture of all amino acids, substantially as described in Example 1, excluding C and W.

The CDK5 consensus substrate motif degenerate peptide library was coupled to KLH and injected into rabbits as described above in

Example 1. Antisera from the most promising rabbit was purified over protein A and adsorbed over an unmodified (nonphospho) CDK5 consensus motif peptide library column. The flow-through of this column was applied to a modified (phospho) – CDK5 kinase consensus motif column eluted at low pH, dialyzed and tested for phosphospecificity. Initial results indicate these antibodies are phospho-specific (data not shown), and it is anticipated that further specificity testing by ELISA, as outlined in Example 1, will indicate these motif-specific, context-independent antibodies are highly specific for the phosphorylated form of the PKC Zeta consensus substrate motif, and will specifically recognize a plurality of different proteins within a genome that contain this motif.

D. Insulin Receptor Consensus Substrate Motif

Insulin binding to the α subunits of the insulin receptor (IR) activates its tyrosine kinase activity in β subunits. Insulin receptor kinase phosphorylates its substrates at a short consensus motif, Y*MXM. Context-independent antibodies specific for the IR consensus substrate motif, Y*MXM, were produced by synthesizing the following biased degenerate peptide library: CXXX(E/D)(E/D)(E/D)Y***MM**FXX (SEQ ID NO: 101), where Y* is phosphotyrosine and bold indicates invariant motif residues, X represents any amino acid excluding cysteine (C) and tryptophan (W), and underlined residues, E/D, M, and F = degenerate positions at which the representation of amino acids is biased to 50% of the underlined residue(s) (glutamic acid/aspartic acid (E/D), methionine (M) or phenylalanine (F), respectively, where E/D means the 50% bias at that position is to E and D collectively) and 50% of all other amino acids excluding cysteine and tryptophan. Bias towards these particular residues at the given degenerate positions was chosen based upon the prior determination of flanking residues (in peptide substrates) favored by insulin receptor kinases, substantially as described above.

The degenerate peptide library was synthesized by standard F-Moc solid phase peptide synthesis using an ABI peptide synthesizer and amino acid mixtures (further excluding tryptophan (W)), substantially as described in Example I. However, for the biased degenerate positions indicated, the mixtures of amino acids used during degenerate coupling reactions were as follows: 50% of the coupling mixture comprised the biased flanking residue (M or F, respectively, or in the case of E/D, 50% of the coupling mixture comprised E and D collectively (*i.e.* 25% bias for each of E and D) and the other 50% comprised a mixture of all amino acids, substantially as described in Example 1, excluding C and W.

The IR consensus substrate motif degenerate peptide library was coupled to KLH and injected into rabbits as described above in Example 1. Antisera from the most promising rabbit was purified over protein A and adsorbed over an unmodified (nonphospho) IR consensus substrate motif peptide library column. The flow-through of this column was applied to a modified (phospho) – IR consensus motif column eluted at low pH, dialyzed and tested for phosphospecificity. Initial results indicate these antibodies are phospho-specific (data not shown), and it is anticipated that further specificity testing by ELISA, as outlined in Example 1, will indicate these motif-specific, context-independent antibodies are highly specific for the phosphorylated form of the all or part of the IR consensus substrate motif, and will specifically recognize a plurality of different proteins within a genome that contain this motif.

25 E. PI3K P85 Binding Motif

PI3K (phosphoinositide-3 kinase) is a heterodimer consisting of a P85 adaptor subunit a P110 catalytic subunit. PI3K is important to signaling by protein-tyrosine kinase receptors. PI3K uses SH₂ domains within the p85 subunit to bind the tyrosine phosphorylated motif, Y*MXM. Context-independent antibodies specific for the PI3K P85 consensus

substrate motif, Y*MXM (SEQ ID NO: 102), were produced by synthesizing the following degenerate peptide library:
CXXXXXXEY*MXMXXX (SEQ ID NO: 103), where Y* is phosphotyrosine and bold indicates invariant motif residues, X represents any amino acid excluding cysteine (C) and tryptophan (W), and the underlined residue, E, is a degenerate position at which the representation of amino acids is biased to 50% of E, and) and 50% of all other amino acids excluding cysteine and tryptophan. Bias towards this particular residue at the given degenerate position was chosen based upon the prior determination of flanking residues (in peptide substrates) favored by PI3K P85 binding, substantially as described above.

The degenerate peptide library was synthesized by standard F-Moc solid phase peptide synthesis using an ABI peptide synthesizer and amino acid mixtures (further excluding tryptophan (W)) for coupling reactions, substantially as described in Example I.

The PI3K P85 binding motif degenerate peptide library was coupled to KLH and injected into rabbits as described above in Example 1. Antisera from the most promising rabbit was purified over protein A and adsorbed over an unmodified (nonphospho) PI3K P85 binding motif peptide library column. The flow-through of this column was applied to a modified (phospho) – PI3K P85 binding motif column eluted at low pH, dialyzed and tested for phosphospecificity. Initial results indicate these antibodies are phospho-specific (data not shown), and it is anticipated that further specificity testing by ELISA, as outlined in Example 1, will indicate these motif-specific, context-independent antibodies are highly specific for the phosphorylated form of the PI3K P85 binding motif, and will specifically recognize a plurality of different proteins within a genome that contain this motif.

F. CaMKII Consensus Substrate Motif

CaMKII (calcium/calmodulin-dependent kinase II) is a kinase that phosphorylates its substrates at a short consensus motif, RQXT*FD.

- 5 Context-independent antibodies specific for the CaMKII consensus substrate motif, RQXT*FD (SEQ ID NO: 104), were produced by synthesizing the following biased degenerate peptide library: CXX**K**RQQ**T***FDLFXXX (SEQ ID NO: 105), where T* is phosphothreonine and bold indicates invariant motif residues, X represents any amino acid
10 excluding cysteine (C) and tryptophan, and underlined residues, K, Q, L, and F = degenerate positions at which the representation of amino acids is biased to 50% of the underlined residue(s) (lysine (L), glutamine (Q), leucine (L), or phenylalanine (F), respectively) and 50% of all other amino acids excluding cysteine and tryptophan. Bias towards these particular
15 residues at the given degenerate positions was chosen based upon the prior determination of flanking residues (in peptide substrates) favored by CaMKII kinase, substantially as described above.

- The degenerate peptide library was synthesized by standard F-Moc solid phase peptide synthesis using an ABI peptide synthesizer and
20 amino acid mixtures (further excluding tryptophan (W)), substantially as described in Example I. However, for the biased degenerate positions indicated, the mixtures of amino acids used during degenerate coupling reactions were as follows: 50% of the coupling mixture comprised the biased flanking residue (K, Q, L, or F, respectively) and the other 50%
25 comprised a mixture of all amino acids, substantially as described in Example 1, excluding C and W.

The CaMKII consensus substrate motif degenerate peptide library was coupled to KLH and injected into rabbits as described above in Example 1. Antisera from the most promising rabbit was purified over

protein A and adsorbed over an unmodified (nonphospho) CaMKII consensus motif peptide library column. The flow-through of this column was applied to a modified (phospho) – CaMKII consensus motif column eluted at low pH, dialyzed and tested for phosphospecificity. Initial results indicate these antibodies are phospho-specific (data not shown), and it is anticipated that further specificity testing by ELISA, as outlined in Example 1, will indicate these motif-specific, context-independent antibodies are highly specific for the phosphorylated form of all or part of the CaMKII consensus substrate motif, and will specifically recognize a plurality of different proteins within a genome that contain this motif.

G. Src Consensus Substrate Motif

Src is a kinase that phosphorylates its substrates at a short consensus motif, EXIY*GEF (SEQ ID NO: 106). Context-independent antibodies specific for the SRC consensus substrate motif, EXIY*GEF, were produced by synthesizing the following biased degenerate peptide library: CXXXEEIY*GEFXXXX (SEQ ID NO: 107), where Y* is phosphotyrosine and bold indicates invariant motif residues, X represents any amino acid excluding cysteine (C) and tryptophan, and underlined residues, E, = degenerate positions at which the representation of amino acids is biased to 50% of the underlined residue(s) (glutamic acid (E)) and 50% of all other amino acids excluding cysteine and tryptophan. Bias towards these particular residues at the given degenerate positions was chosen based upon the prior determination of flanking residues (in peptide substrates) favored by SRC kinase, substantially as described above.

The degenerate peptide library was synthesized by standard F-Moc solid phase peptide synthesis using an ABI peptide synthesizer and amino acid mixtures (further excluding tryptophan (W)), substantially as

described in Example 1. However, for the biased degenerate positions indicated, the mixtures of amino acids used during degenerate coupling reactions were as follows: 50% of the coupling mixture comprised the biased flanking residue (E) and the other 50% comprised a mixture of all amino acids, substantially as described in Example 1, excluding C and W.

The SRC consensus substrate motif degenerate peptide library was coupled to KLH and injected into rabbits as described above in Example 1. Antisera from the most promising rabbit was purified over protein A and adsorbed over an unmodified (nonphospho) SRC consensus motif peptide library column. The flow-through of this column was applied to a modified (phospho) – SRC consensus motif column eluted at low pH, dialyzed and tested for phosphospecificity. Initial results indicate these antibodies are phospho-specific (data not shown), and it is anticipated that further specificity testing by ELISA, as outlined in Example 1, will indicate these motif-specific, context-independent antibodies are highly specific for the phosphorylated form of the SRC consensus substrate motif, and will specifically recognize a plurality of different proteins within a genome that contain this motif.

20

H. CDC2/CDK2 Consensus Substrate Motif

CDC2/CDK2 (cell division cycle protein 2/cyclin dependent kinase 2) is a kinase that phosphorylates its substrates at a short consensus motif, S*PR(K/R) (SEQ ID NO: 108). Context-independent antibodies specific for the CDC2/CDK2 consensus substrate motif, S*PR(K/R), were produced by synthesizing the following biased degenerate peptide library: CXXXHHH(K/R)**S*PR(K/R)**RXXX (SEQ ID NO: 109), where S* is phosphoserine and bold indicates invariant motif residues (K/R indicating either K or R must be present at that position), X represents any amino

acid excluding cysteine (C) and tryptophan, and underlined residues, H, K/R, and R = degenerate positions at which the representation of amino acids is biased to 50% of the underlined residue(s) (histidine (H), lysine/arginine (K/R), or arginine (R), respectively, where K/R means the 50% bias at that position is to K and R collectively) and 50% of all other amino acids excluding cysteine and tryptophan. Bias towards these particular residues at the given degenerate positions was chosen based upon the prior determination of flanking residues (in peptide substrates) favored by CDC2/CDK2 kinase, substantially as described above.

10 The degenerate peptide library was synthesized by standard F-Moc solid phase peptide synthesis using an ABI peptide synthesizer and amino acid mixtures (further excluding tryptophan (W)), substantially as described in Example I. However, for the biased degenerate positions indicated, the mixtures of amino acids used during degenerate coupling
15 reactions were as follows: 50% of the coupling mixture comprised the biased flanking residue (H or R, respectively, or in the case of K/R, 50% of the coupling mixture comprised K and R collectively (*i.e.* 25% bias for each of K and R) and the other 50% comprised a mixture of all amino acids, substantially as described in Example 1, excluding C and W.

20 The CDC2/CDK2 consensus substrate motif degenerate peptide library was coupled to KLH and injected into rabbits as described above in Example 1. Antisera from the most promising rabbit was purified over protein A and adsorbed over an unmodified (nonphospho) CDC2/CDK2 consensus motif peptide library column. The flow-through of this column
25 was applied to a modified (phospho) – CDC2/CDK2 consensus motif column eluted at low pH, dialyzed and tested for phosphospecificity. Initial results indicate these antibodies are phospho-specific (data not shown), and it is anticipated that further specificity testing by ELISA, as outlined in Example 1, will indicate these motif-specific, context-
30 independent antibodies are highly specific for the phosphorylated form of

all or part of the CDC2/CDK2 consensus substrate motif, and will specifically recognize a plurality of different proteins within a genome that contain this motif.

5 I. GSK3 Consensus Substrate Motif

GSK3 (glycogen synthase kinase-3) is a kinase that phosphorylates its substrates at a short consensus motif, T*PXXS*P. Context-independent antibodies specific for the GSK3 consensus substrate motif, T*PXXS*P (SEQ ID NO: 110), were produced by synthesizing the following biased degenerate peptide library:

10 CX(P/F)X(P/L)(P/L)PT***PP**(P/L)**S***PXXXXX (SEQ ID NO: 111), where T* is phosphothreonine, S* is phosphoserine and bold indicates invariant motif residues, X represents any amino acid excluding cysteine (C) and tryptophan, and underlined residues, P/F, P/L, and P = degenerate

15 positions at which the representation of amino acids is biased to 50% of the underlined residue(s) (proline/phenylalanine (P/F), proline/leucine (P/L), or proline (P), respectively, where P/F and P/L mean the 50% bias at that position is to P and F, or P and L, collectively) and 50% of all other amino acids excluding cysteine and tryptophan. Bias towards these

20 particular residues at the given degenerate positions was chosen based upon the prior determination of flanking residues (in peptide substrates) favored by GSK3 kinase, substantially as described above.

The degenerate peptide library was synthesized by standard F-Moc solid phase peptide synthesis using an ABI peptide synthesizer and

25 amino acid mixtures (further excluding tryptophan (W)), substantially as described in Example I. However, for the biased degenerate positions indicated, the mixtures of amino acids used during degenerate coupling reactions were as follows: 50% of the coupling mixture comprised the biased flanking residue (P, or in the case of P/F and P/L, 50% of the

coupling mixture comprised P and F, or P and L, collectively (*i.e.* 25% bias for each of P and F or L) and the other 50% comprised a mixture of all amino acids, substantially as described in Example 1, excluding C and W.

- 5 The GSK3 consensus substrate motif degenerate peptide library was coupled to KLH and injected into rabbits as described above in Example 1. Antisera from the most promising rabbit was purified over protein A and adsorbed over an unmodified (nonphospho)GSK3 consensus motif peptide library column. The flow-through of this column
- 10 was applied to a modified (phospho) – GSK3 consensus motif column eluted at low pH, dialyzed and tested for phosphospecificity. Initial results indicate these antibodies are phospho-specific (data not shown), and it is anticipated that further specificity testing by ELISA, as outlined in
- 15 Example 1, will indicate these motif-specific, context-independent antibodies are highly specific for the phosphorylated form of the GSK3 consensus substrate motif, and will specifically recognize a plurality of different proteins within a genome that contain this motif.

J. Proline-Phosphoserine-Proline Motif

- 20 Some important signaling proteins can be regulated by phosphorylation at a serine between two adjacent prolines. For example, GSK3 phosphorylates its substrates at T*P or S*P preceded preferentially by proline. P53 and L-myc also contain a phosphorylated serine between two prolines. Context-independent antibodies specific for this motif, PS*P, were produced by synthesizing the following degenerate peptide
- 25 library: CXXXXX**PS*P**XXXXXX (SEQ ID NO: 112), where S* is phosphoserine and bold indicates invariant motif residues, and X represents any amino acid excluding cysteine (C) and tryptophan. No bias was introduced at any of the degenerate residues flanking the motif.

The degenerate peptide library was synthesized by standard F-Moc solid phase peptide synthesis using an ABI peptide synthesizer and amino acid mixtures (further excluding tryptophan (W)) for coupling reactions, substantially as described in Example I.

- 5 The PS*P motif degenerate peptide library was coupled to KLH and injected into rabbits as described above in Example 1. Antisera from the most promising rabbit was purified over protein A and adsorbed over an unmodified (nonphospho) serine peptide library column. The flow-through of this column was applied to a modified (phospho) – PS*P
10 binding motif column eluted at low pH, dialyzed and tested for phosphospecificity. Initial results indicate these antibodies are phospho-specific (data not shown), and it is anticipated that further specificity testing by ELISA, as outlined in Example 1, will indicate these motif-specific, context-independent antibodies are highly specific for the
15 phosphorylated form of the PS*P motif, and will specifically recognize a plurality of different proteins within a genome that contain this motif.